BIOLOGICAL REVIEWS

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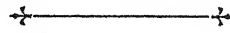
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VOLUME III
1928

CONTENTS

No. 1, January 1928

No. 2, March 1928

No. 3, July 1928

No. 4, October 1928

INDEX OF AUTHORS

ERRATA

Page 49, line 16. *For* labial *read* labral.

„ 50, „ 1. *For* labial *read* labral.

„ 53, „ 4 of footnote. *For* gnathobases *read* gnathobasæ setae.

THE MERISTEMATIC TISSUES OF THE PLANT
BY J. H. PRIESTLEY.

(*Received* June 17, 1927.)

(With Eight Text-figures.)

CONTENTS.

INTRODUCTION.

SCHÜEPP (1926) in a valuable and recent monograph, has given a detailed analysis of the data and a thorough review of the literature in relation to the meristem and its activities. With this monograph as a basis it is possible, in relatively brief compass, to review the subject from a "causal" standpoint, that is to show each stage in development as dependent on preceding events, and as releasing in its turn a system of internal correlating factors which control the continued progress of growth. The behaviour of this internal system is, to some extent, modifiable by environment so that the plant is the result of a finely balanced system of external and internal factors, working within a wide range of possibilities, limited by the nature of the material which heredity has provided.

Such a view of the existence and activities of a meristem is attempted in the following pages, and the necessary rearrangement of the data which is entailed, and the new correlations so produced, may suggest fresh lines of experimental attack for the investigation of one of the fundamental processes of the living and developing plant.

A developmental study of the meristem necessitates a knowledge of the physiology of the living cells for which we are still insufficiently equipped, so that some conclusions are drawn on inadequate grounds. Such weaknesses are fully emphasised and justification must lie in the more coherent presentation of the perplexing and, from other points of view, seemingly unrelated phenomena.

There is no attempt to make bibliographic treatment complete, and such citations of literature as are given must be regarded as supplementary to the bibliography to be found at the end of Schüepp's monograph.

THE APICAL MERISTEMS.

It is well known that in all the vascular plants, the meristematic, actively growing tissues of the plant are localised to certain well defined regions. In some, such as the majority of the monocotyledons, all the adult complexities of shoot and root may be traced to two apical regions, the growing points of shoot and root, and in Dicotyledons and Gymnosperms, although the increase in girth of the axis is due to the activity of differently situated though still well defined growing regions, yet the original organisation of shoot and root, the distribution of what are sometimes termed the primary tissues, is again the result of the activity of such apical growing points. The developmental problem has therefore one great initial advantage, it admits of strict localisation. The first step to the understanding of the organisation of the adult plant body is a knowledge of the manner in which it is produced as the result of the activity of these apical growing points.

All higher plants are cellular organisms, and the growth centres are centres where cells, with all the potentialities of development, multiply as the result of a continual synthesis of living protoplasm. These growing tissues are described as meristematic and their apical groups of growing cells as the apical meristems. The first problem, therefore, is to record the available data as to the apical meristems and to see if any elucidation is possible of the many and varied tissues that follow as a result of their activity. This will provide a basis from which to interpret to some extent the other meristematic activities of the plant, including those responsible for the intercalary increase in girth.

THE EVOLUTIONARY HISTORY OF THE APICAL MERISTEM.

It is proposed to confine this study in the main to the more highly organised flowering plants, but a very brief review of the steps in the differentiation of these apical growing regions, as illustrated in existing groups of plants, is not lacking in suggestiveness.

In the lowly organised, autotrophic plants, as the filamentous alga *Spirogyra*, there is no differentiation between growing cell and adult cell; all cells alike are active in constructive metabolism, in photosynthesis, and all the cells construct protoplasm, grow and ultimately multiply by division. In the filamentous plants, however, growth may be restricted to some region of the filament, whilst on the other hand the remaining cells may be conspicuously active in photosynthesis, the food material thus obtained being presumably, in part, made available by translocation for the subsistence of the actively growing regions. Filamentous organisms, such as are found in the Siphonales, where cross walls are infrequent, probably oppose very slight resistance to such translocation and possibly this is a contributary cause to the localisation of growth which is seen in this group.

Noll's (1903) observation of the localisation of the growing region to the apex in this group is particularly significant. He points out that the protoplasmic mass is in active streaming motion, the movement persisting through the growing region, in and out of which individual protoplasmic units are constantly moving. The localisation of growth here therefore is not to a particular group of protoplasmic units, but is determined in some way by the general organisation of the whole plant body. That part of the protoplasmic mass which finds itself, as the result of position, for the time being in a particular relation to the internal organisation of the plant, becomes active in protoplasmic synthesis and "meristematic," because although new cells are not cut off, new nuclei are formed by division and act as new centres of protoplasmic activity (energids).

The contribution of the differentiated multicellular growing regions to growth may be regarded as threefold: (1) synthesis of new protoplasm, (2) the formation of new centres of protoplasmic activity, usually new cells, by division, (3) extension in size, partly by increase in protoplasmic mass, but usually by the more evident process when, following usually on the cessation of protoplasmic synthesis (see p. 5), the volume of the cell increases as the result of the intake of water upon vacuolation. When a special growing region is differentiated in the lower groups of plants, its activity in respect to those different processes may vary from that which is characteristic of the higher plants.

In the Sphacelariales, for instance, growth is restricted to the apical cell. When a cell is cut off by a wall at the base of the apical cell, growth is now restricted to the cell above the new wall; the cell below in turn divides a number of times but no further increase in mass takes place (Fig. 1). In the vast majority of more highly organised Thallophyta, the growing region is characterised by the possession of an apical cell, which is itself actively growing and which is characterised by its shape, size and manner of division. In this cell there is an equilibrium between the processes of protoplasmic increase and cell division of such a nature that after each cell division, the apical cell returns to its original size and shape before another division takes place. In practically every case this apical cell is larger than the segments which are cut off from it. The cells so cut off may continue, however, to grow and divide for a time, but the orderly succession of walls thus arising does not destroy the symmetry of the mass, which is such that the adult tissue ultimately formed is clearly referable in origin to the divisions which originally took place in the apical cell.

Fig. 1. Growing apex of *Cladostephus verticillatus* (after Oltmanns).

The large size of the apical cell deserves further analysis. The cell is usually full of protoplasm and unvacuolated, so that the absence of interior walls in this relatively large mass means that its synthetic activity is restricted to the synthesis of protein and protoplasm. The protoplasmic mass below, more intersected with walls, must proportionally be forming relatively more carbohydrates in addition

to the new protoplasm. In a sense then, the apical cell is more strictly concerned with the primary process of growth—the synthesis of protoplasm—but this may be at some expense of efficiency because the protoplasmic cell mass must receive continually supplies of food from which to construct protoplasm. These supplies must be taken in through the surface of the protoplast and the larger mass of the apical cell means a smaller relative surface through which these supplies are received for the use of the protoplasmic mass, which is throughout engaged in synthetic metabolism. Carbohydrate walls dividing the protoplasmic mass create new protoplasmic surfaces to which supplies may penetrate along the wall. The smaller cells behind the apical cell, therefore, if they remain meristematic, may be more adequately nourished and may grow more rapidly than the apical cell itself, a phenomenon which is often illustrated in the organisation of the growing region around the apical cell. Schüepp points out that a diminution in size of the meristematic cells just behind the apex, coupled with greater growth activity, is widespread throughout the higher Cryptogams.

Schüepp also points out that in other plants, perhaps more highly organised, although the single apical cell can no longer be distinguished, the apex of the growing region is crowned with a few relatively large cells from which, as the subsequent division planes suggest, all the surrounding tissue can be traced. These cells are not apical cells because they have not the characteristic shape and manner of division and Schüepp suggests that they should be termed initial cells. It will be seen that their outstanding character is their size, as compared with that of neighbouring meristematic cells, and this means that they are again cells in which protoplasmic synthesis predominates at the expense of the carbohydrate synthesis which is dividing the neighbouring cell into smaller and possibly more efficient units through the more frequent deposition of walls. This question of efficiency is speculative, but it is at least very suggestive in that while such initial apical cells are found in the Lycopodiales and Gymnosperms and seem to be replacing apical cells in the Eusporangiate Ferns (Bower, 1889–90); in the Angiosperms, with very few exceptions (*e.g.* Elodea), the apical meristem consists of a uniformly small celled tissue in which no large initial cell can be distinguished and the volume of the meristem cells is minimal compared with the volume of the cells of any surrounding tissue.

THE MERISTEM CELL IN THE HIGHER PLANTS.

Singularly little can be said as to the detailed cytology of the meristem cell of the higher plant, possibly because its structural organisation can yield little upon investigation by present methods.

Its most striking feature is the uniformity with which the typical meristem cell is reported as having no recognisable structural features beyond cytoplasm and nucleus.

The nucleus has all the characteristic structural features—the nucleus of the root growing point in particular being one of the most frequent sources of cytological data as to nucleolar behaviour, details of mitosis, etc.—but the cytoplasm

seems singularly devoid of any definite structural units. Neither plastids nor vacuoles are reported as present in this cytoplasm, nor any granules of reserve food substance, such as starch, oil or protein granules. With the newer technique, using metal salts, etc., chondriosomes are being described and the genetic connection of these with the plastids and vacuoles that arise in the cells derived from the meristem at a very early stage is still a subject of controversy. To summarise this literature would take too much space and on the most essential point there seems to be agreement, viz., that visible structural differentiation is at a minimum in the cytoplasm of the meristem cell. It is not suggested that the cytoplasm is less complex in reality, because these cells are actively engaged in the complex constructive metabolism involved in protoplasmic syntheses. This cytoplasm does, however, seem to be devoid of structural detail in particular of solid bodies with definite structural outline, and it seems possible, indeed, that the cytoplasm as a whole is frequently in the sol condition, even if during the process of cell division, it passes temporarily at frequent intervals into a more gel-like state. The reason for suggesting this fluid nature for the cell with resting nucleus is the uniformly spherical outline of the nucleus in all apical meristems. From the cytological standpoint, the cytoplasm of the meristem cell in fixed and stained preparation might often be described as interspersed with very fine vacuoles. These vacuoles are, however, of a different order to those present in the "vacuolated cell." They are perhaps evidence, so far as fixed stained material can supply it, that the living cytoplasm is not a homogeneous one-phase system but contains different phases of different density and composition. There seem, however, to be no vacuoles charged with vacuolar sap containing solutes exerting osmotic pressure so that the liquid contents of the vacuole exert a hydrostatic pressure, driving the protoplasmic contents against the wall of the cell and the cell wall against its neighbours. There may of course be definite osmotic pressure due to dissolved substances in the sol phase of the protoplasm itself. This absence of vacuoles, compared with the rapid expansion of the vacuolated cells in the immediate neighbourhood of the meristem, certainly suggests further that the internal expansive forces at work in the meristem cells are less than those at work in their neighbours, with the result that the meristem cells may be regarded as receiving their shape owing to their growth as plastic units against a certain amount of external pressure. The meristem cells can be separated by suitable process of maceration (Tupper-Carey and Priestley, 1924). They seem to be somewhat irregular, many sided bodies, often showing an approximation to the tetrakaidekahedral figure which might be anticipated if the pressure upon them from all sides were uniform. The walls bounding the cells are comparatively thin, but when stained with suitable methods after maceration, the cellulose is found to be by no means uniformly distributed, but is more thickly deposited at the angles of the cell. Between cell and cell is a very thin matrix, probably of protein and pectin (Tupper-Carey and Priestley, 1923), but not of salts of pectic acid, which can be dissolved away by suitable alkaline reagents. This substance is by no means a hard cementing material but is probably sufficiently plastic to keep the cells in continuous contact as the result

of the pressure exerted by the surrounding vacuolating tissues. The result is that the cells may be described as possessing cellulose and pectin walls, with a middle lamella of protein-pectin, in continuous contact. No air spaces are present. On the other hand, as soon as the cell vacuolates, the internal pressure, acting on the elastic wall, tends to change its shape from the regular outline received under external pressure to a spherical shape conforming to the internal hydrostatic pressure. The plastic middle lamella offers insufficient resistance to these tendencies and the cells draw away from one another at the corners, with the result that intercellular air spaces arise in association with vacuolation, the middle lamella subsequently hardening with the deposition of calcium pectate.

The change from the typical meristematic cell to this normal vacuolated parenchyma cell is naturally very gradual and frequently admits of a distinction into certain stages. Thus Schüepp recognises the existence, in addition to the typical "embryonic meristem" cell just described, of larger cells of a "primary" or "half-meristem" type which contain vacuoles but which still divide though less frequently.

The writer has also drawn attention to the fact that in the higher plants, in many cases, a zone of cells can be distinguished between the typical meristem and the vacuolated cells, which are themselves not vacuolated but contain solid inclusions such as starch grains, although the starch is frequently so masked (probably associated with protein) as only to react with iodine reagents after appropriate preliminary treatment (Priestley, 1926 a). The existence of such non-protoplasmic granules, like the formation of a carbohydrate wall at the surface of the protoplast itself, is an indication of the gradual transference of constructive metabolic activity from the construction of protoplasm, the primary business of the meristematic cell, to the condensation of anhydrides of carbohydrates. Undoubtedly with attention directed to the point, more data will accumulate as to the distinctions between the true meristematic cell and the differentiating cells derived from them. One difference of importance which was described by Rosen in 1896 for Oleander, has been confirmed by Hof in 1898 for Pteris, and has often been noted by the writer in his studies of vegetative propagation, is that meristematic tissues behave differently under double staining from the adult tissues around them. Rosen describes the "cyanophil" reaction of the nuclei in the meristematic cells as compared with the "erythrophil" of the nuclei in the vacuolating and differentiated cells. Put more generally, the nuclei of the meristem show a different permeability to stains and a different power of retaining them even in fixed material. This behaviour will require considerably more examination before its elucidation is possible, but it is certainly suggestive of a different hydrogen ion reaction in the cytoplasmic nuclear interspace, which has left its impression upon the reactivity of the protein constituents of the nucleus.

THE ORGANISATION OF THE APICAL MERISTEM.

The organisation of adult shoot and root in the flowering plant is strikingly different and these differences must be inherent in the apical meristems from which these tissues are derived, as, with very rare exceptions, no modification of external environment will produce from shoot and root apices anything but shoot and root respectively. Quite possibly any individual meristem cell, at either shoot or root apex, has equivalent potentialities. The facts of regeneration, where isolated pieces of shoot prove capable of regenerating roots, and *vice versa*, point
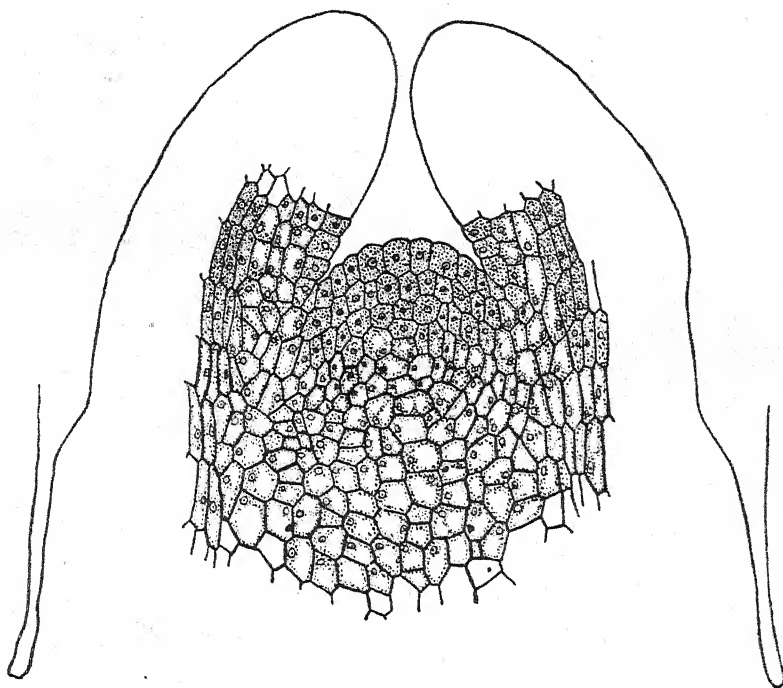
Fig. 2. Growing-point of *Vinca minor* in longitudinal section. Meristem cells (stippled to centre of cell) cover the apex of the shoot and the lateral folds which form youngest pair of leaves.

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in this direction. But in the organised shoot or root apex, the *organisation* is already different and definitely determines future development. So that, apart from Beijerinck's observation (1886) of the production of a root from a shoot bud on the root of *Rumex Acetosella*, there are no clear cases in the literature of a reversal of this trend of development when once settled by the organisation of a growing apex.

A difference in the organisation of the meristems of shoot and root is, therefore, to be expected and is apparent in all vascular plants. The following notes present a partial analysis of these differences in the case of the flowering plants. Here the

tissue active in protoplasmic synthesis and cell division is always composed of small cells. Throughout a very wide range of plant forms the size of these

meristem cells varies very little and there can be little doubt that it represents a suitable balance between mass of cell protoplasm engaged in synthesis and cell surface through which the necessary food materials are absorbed. The difference in organisation between shoot and root is shown clearly in the distribution of these similar meristematic cells. *In the shoot* they are always at the surface of the apex, lying just beneath a thin but definite cuticle which covers the whole apex (Fig. 2). This layer of meristem varies in depth from species to species. Sometimes it is only one or two cells deep, in other cases many celled; in the same species it may be modified to some extent by environmental conditions, for instance, growth in darkness is associated with a greater depth of meristem (Priestley, 1926 *a*). As Schüepp has shown in certain cases (1914) the rate of cell division is practically the same in all layers of the meristem from the surface inward, judging from the proportion of cells which may be found in some stage of division at any depth. In the surface layer new cells are always added to it by the septation by anticlinal wall of cells already in this layer, on the other hand in deeper cell layers new cells are cut off in all three directions from the original cells. Schüepp points out that this state of affairs can only have one result—a great increase of surface upon the central core as growth continues. This seems to be the fundamental basis of the formation of the surface folds which arise at the shoot apex as new leaf and branch initial. The exact order of succession of these folds is constant for the species, and is a feature
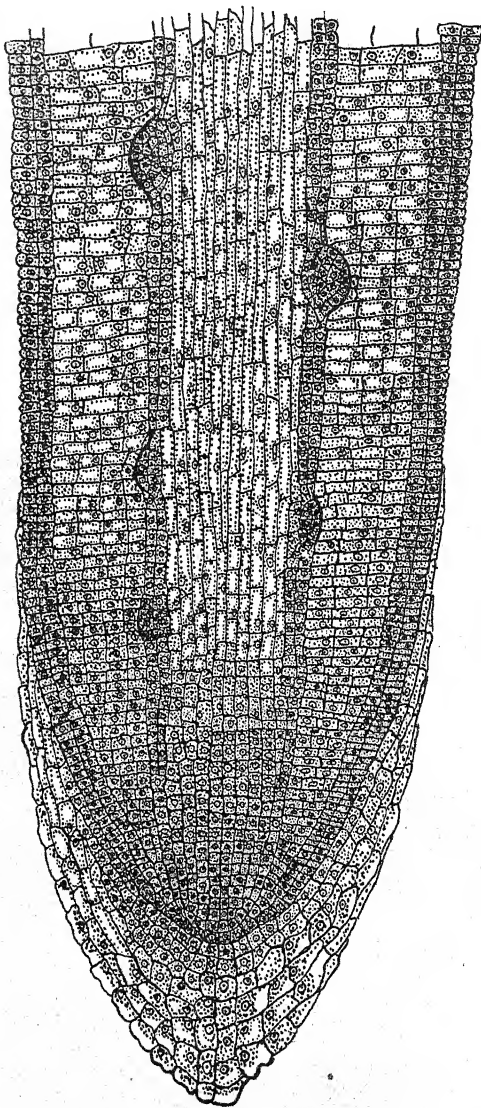

Fig. 3. Root of Arum Lily in longitudinal section. The meristematic tissue is sunk below the root cap cells, which are vacuolated. Young branch root initials are appearing in the meristematic pericycle.

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of organisation which is inherited, but the developmental machinery which permits its expression seems to be at bottom the simple relations of the cell divisions in the different meristematic layers which will be further examined in the next section.

In the root apex similar meristematic cells are never found at the surface, which is clothed with the vacuolated cells of the root cap in which cell division has ceased, but behind these, and therefore buried beneath the surface, is found a dense mass of meristematic cells (Fig. 3).

Towards the apex these cells are dividing slowly and adding cells to the cap, usually at about the rate at which the cap cells are being lost at the surface by abrasion or other processes. Passing inwards from the outermost layer, the meristem cells will be found to be dividing more quickly, the cells lying in filament-like rows —"Rippenmeristem" (Schüepp, 1926), because division walls occur most frequently in the plane transverse to the root axis. Such a system of cell increase can only have one result—increase in length without any additional extension of surface, and the root apex is never clothed with folds of any type, young root initials arising further behind the apex of the root as endogenous outgrowths.

The origin of such fundamental differences in organisation of shoot and root apex must be deep seated. An attempt to trace the causation led to the discovery that the walls intervening between the meristem cells at these apices are very different in nature (Tupper-Carey and Priestley, 1923). At the shoot apex the cellulose reaction is given directly with iodine reagents after hydrolysis and swelling with critical strength sulphuric acid (about 70 per cent.). The iodine reagent is not successful after swelling with concentrated aqueous zinc chloride unless the sections are first treated with cold aqueous potash. In the root, however, no cellulose reaction with iodine follows, after any hydrolysing agents, unless the sections are first boiled with either alcoholic or aqueous potash. Reasons have been given for concluding that the difference depends upon the nature of the substances other than cellulose in the wall and the extent to which they are present. In the root it is concluded that both protein and fatty substances are largely present in the walls, in other words, probably the protoplasm of the living cell is still interpenetrating the carbohydrate matrix. On the other hand, save for traces removable by immersion in cold alkali, such substances appear to be absent in the walls of the shoot apex.

The relation between these differences in reaction to cellulose reagents and the organisation of the meristematic tissue is still hypothetical, but the following argument has been tentatively advanced and does not seem an undue forcing of the few facts available. It is clear that meristematic activity, involving protoplasmic synthesis, must be absolutely dependent upon a continuous supply of food material from which protoplasm may be constructed. This food material undoubtedly comes in some manner from the vascular system, whether it be from phloem or xylem. Both these tissues end, in both root and shoot apex, some distance below the region where active meristematic growth is in progress. However these nutrients may then penetrate through the vacuolated tissue intervening, the indi-

vidual meristematic protoplast is undoubtedly receiving this food supply through its surface from the wall, and there can be no doubt whatever that in the meristematic region the carbohydrate walls cleaving the meristematic mass are of the first importance, as the channels along which these food materials reach the centres of protoplasmic synthesis. But it is clear that a wall which is nearly pure carbohydrate, as in the shoot, will permit of readier diffusion than a similar wall in which the space available between the carbohydrate molecules is filled with substances of a protoplasmic nature—as in the root.

Thus in the shoot, food materials will be making their way through the wall to the very surface of the meristem, and the surface layers will be growing and dividing as rapidly as the deeper layers, with the consequent production of the superficial folds. In the root the supplies make their way more slowly outwards and the outermost meristematic cells are dividing more slowly than the inner, so that growth is in length without the production of folds at the surface.

FACTORS CONTROLLING CELL DIVISION IN THE MERISTEM.

The cells of the apical meristem in the Flowering Plant only vary in size within a comparatively narrow range. This is clear evidence that increase in mass is always followed by cell division before certain limits of size are exceeded. Quite possibly, the increased mass itself in some way determines the operation of the division mechanism. This is speculative, as at present are all suggestions as to factors controlling cell division, but two of the commonest generalisations about cell division seem to be of great interest if applied to the organisation of the apical meristem.

The first generalisation, sometimes termed Sachs' Law, states that when a protoplasmic mass divides into two, the two daughter cells will be equal in mass. Experience shows that this rule generally applies provided there are no special local accumulations of reserve substances in any part of the cell. In cells such as are found in an apical meristem, where the whole mass of cytoplasm seems remarkably free from any special, local structural features, and where the whole protoplasmic mass seems engaged in the process of protoplasmic synthesis, this law should apply, and does so, as far as can be judged from observations based upon the study of microtome sections.

The second generalisation, which is very fully discussed by D'Arcy Thompson (1917, *loc. cit.* Chaps. VII and VIII), may be cited here as Errera's Law. It states that when such a cell division takes place, if the dividing cell is in equilibrium with its external surroundings, the semi-liquid dividing wall tends to be of minimum area. The application of this generalisation throws quite a new light upon the organisation of the cells of the shoot apex.

These have usually been classified, following Hanstein, into three layers, dermatogen, periblem and plerome. These layers were characterised by Hanstein, by the behaviour of the actively dividing cells. The outermost layer of the shoot meristem, the dermatogen, is genetically distinct from all layers below, because

owing to the succession of cell division with anticlinal walls, its new cells always arise from division of these outermost cells alone.

Below these are one or more layers of cells distinguished as periblem which are genetically equally distinct because here again over the growing apex, all new walls are anticlinal and all divisions add cells simply in the same plane parallel to the surface. Within the periblem is a core, in which other cell walls seem to form indifferently in all three planes. Cells are, therefore, added to all the thickness of the central core, and this central meristematic mass is distinguished as the plerome. Incidentally, as cells divide as rapidly in periblem as in plerome and dermatogen, it is clear that the superficial folds, the leaf initials and axillary buds will be contributed to both by dermatogen and periblem but not by plerome (see p. 8). Hanstein's original distinctions of these different tissues were based upon these differences in position and in their behaviour during growth within the meristem itself. Later, with the conclusion that the dermatogen gives rise to the epidermis, the periblem to the cortex and the plerome to the stele, there has been a tendency to a kind of extrapolation from these distinctions based on the adult structure and to look for and define the periblem as the tissue which gives rise to the cortex, etc.

If it is realised that a meristem consists of a plastic mass of cells moulded by external pressures which compress the cells together so that their individual shape is determined by the forces produced by the growing tissues in their neighbourhood, then with the application of Errera's Law, quite new light is thrown upon Hanstein's original definitions. In the shoot apex, the meristem seems to be compressed between the limiting cuticle and external cellulose wall on one side, thin and but little consolidated though these are as yet, and the vacuolated cells beneath them on the other. The result is that the outermost cells of the meristem are not symmetrical 12 or 14-sided figures, but are flattened by the pressure from below, against the outer limiting wall. As a result they tend to have a long axis parallel to the surface with one immediate result, all divisions, if Errera's Law is obeyed, will be at right angles to the surface. And this is exactly the feature of the external meristematic layer which defines Hanstein's dermatogen. If the pressure on the apex is sufficiently great between the expanding tissue below, and the resistant stretched surface above, then other layers will tend to be flattened parallel to the surface also, and thus will arise beneath the dermatogen, a periblem in which the cells also have a long axis parallel to the surface and divide as a consequence continually by walls at right angles to the surface. Further within the tissues, pressures are more balanced, cells more symmetrical in shape, and divisions as a consequence in all three planes, the conditions found in the plerome.

But from this standpoint it might well be doubted whether such differences in division as are utilised by Hanstein in his classical distinction of these three tissues, will constantly be present. Clearly the extent of the pressure at a growing apex will differ with the general organisation and rate of growth of the plant. An apex with tightly stretched meristematic dome and rapid extension of the cells beneath, may have a large number of cell layers extended, parallel to the surface

and therefore with the characteristics of periblem. But if the apical cone has a broad truncate top with small evidences of upward pressure, the cell extension parallel to the surface may not be consistent enough, even in the most external layer, to keep even a dermatogen wall characterised by anticlinal divisions. This is exactly the condition which exists, as Schüepp points out in his monograph.

In Elodea or Hippuris with a very sharp apex, driven up to a steep point by the pressure of the tissues within, the periblem, as defined by Hanstein, consists of many layers (Fig. 5). On the other hand, in the broad apex of the Gymnosperm, not even the dermatogen is always sharply delimited. Schmidt also (1924) in his studies of the apex of many Angiosperm shoots, shows that the periblem as defined by Hanstein may vary from a doubtful one layer to a depth of several (Figs. 4 and 5).
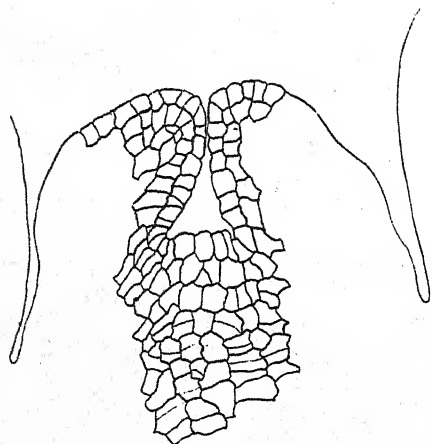
Fig. 4. Median longitudinal section of growing apex of lilac shoot. The periblem, distinguished by anticlinal division walls, is 2 cells deep below the one-layered dermatogen.
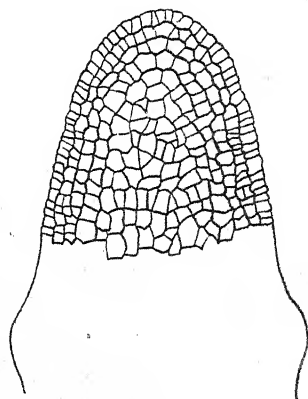
Fig. 5. Tip of shoot apex of *Hippuris vulgaris* in longitudinal sections. The periblem at the apex will be seen to be 4 cells in depth.

This application of Errera's Law to interpret the planes of division in the meristematic cells conforms to a principle laid down by Sachs (1887, *loc. cit.* pp. 430–431). He emphasised the fact that the behaviour of the individual cells in the growing point was determined by their position and the forces of the system of which they formed a part. These general factors, and not the characteristics of the individual cells themselves, determined the behaviour of the cells and thus the future of the growing tissue.

INTERCALARY MERISTEMATIC ACTIVITIES.

The Procambial Ring.

If a series of transverse sections is cut across the meristematic shoot apex with the microtome, it will be found that, when meristematic cells begin to be replaced by vacuolated cells, whilst these appear in the region of pith and cortex, there is an intermediate region where the cells remain full of protoplasm and

meristematic. The cortical and pith cells expand considerably in size upon vacuolation, they also, as they change in shape as they expand in response to internal hydrostatic pressure, move away from one another at the corners, leaving air spaces to which air filters from stomatal apertures now occurring in the differentiated epidermis. The cells in the meristematic ring, however, remain closely adpressed to one another and are obviously under an increased pressure due to the tissue expansions on either side of them. As a result the cells tend to elongate

Fig. 6. Photograph of a transverse section across the young shoot apex of *Vicia faba*. The procambial ring is indicated by the dense contents of the cells, they lie between the larger vacuolated cells of cortex and pith.

now in a longitudinal direction so that the individual cells adopt that shape which is characteristic of the so-called procambial elements.

These procambial elements have usually been described as occurring in isolated groups, the procambial strands, but, as Kostytschew has recently emphasised (1922), they commence below the apex as a continuous ring in the vast majority of Dicotyledons, the subsequent isolation into bundles occurring through development of parenchymatous tissue from part of the ring when in other parts of the ring vascular elements differentiate in connection with the bases of the leaf initials. The procambial ring is broken at intervals in the region of the future node, owing

to a displacement of part of the meristematic ring which moves outwards so as to lie in the centre of the base of the leaf initial. The *shape* of the cells in the procambial ring is thus a result of the pressures to which plastic meristematic elements in this position in the apex are subjected, the reason for their maintenance as meristematic elements amongst vacuolating tissues has still to be found (see p. 18). It must be emphasised that in all cases these procambial elements seem to be in direct genetic continuity with the meristematic tissues at the surface of the stem apex. They soon begin to differ in *size,* however, from the meristematic cells at the apex, the mass of the individual procambial cell being relatively much greater. This is intelligible, because, owing to the steady pressure which determines their growth in length, increased mass is not accompanied by a decreased proportion of surface to mass. They are therefore a further argument for the conclusion that the controlling factor determining the maximum size of a meristematic cell is the relation of surface, through which food reaches it, to mass which utilises this food in protoplasmic synthesis.

In the procambial ring these processes begin to occur at almost the same time, viz. on the inside xylem elements differentiate from some of the procambial elements, on the outside phloem elements; between the two certain of the meristematic cells, besides dividing transversely in accordance with Errera's Law, also divide in a tangential plane, that is a division wall forms in a plane of *maximum* area.

The Cambium.

Divisions in this plane follow one another rapidly all round the median position of the ring and thus a functional vascular cambium is developed. Very frequently divisions in this plane precede all indications of xylem development so that when the first protoxylem elements differentiate they arise in cells which form part of a series arising from the tangential division in the cambium. These elements are thus protoxylem elements, because they are differentiated before stem elongation ceases, and show either annular or spiral lignified thickenings inside extensible cellulose walls. They are, however, secondary xylem elements formed from the cambium and many dicotyledons are without primary xylem, that is xylem elements differentiated from cells which are not in serial relation to the cambium.

The cambium cells themselves, as meristematic elements, show two anomalous features. The first, as I. W. Bailey has emphasised, is the wide departure in these cells from the usual nuclear cytoplasmic ratio found in meristematic cells (1920). The explanation of this probably lies in the fact that *all* the protoplasm of a meristematic cell is engaged in protoplasmic synthesis, not merely the nucleus, the ratio that therefore has to be maintained is that between external surface and total mass, not between external surface and nucleus, or between cytoplasm and nucleus. On the other hand, in vacuolated cells which continue to manufacture protoplasm, as in the cells of *Spirogyra*, there is experimental evidence for connecting protoplasmic synthesis with the (unvacuolated) nucleus, not with the cytoplasm; in

cells growing under these conditions a maintenance of the nuclear cytoplasmic ratio within narrow limits would be understandable.

The other anomaly in the cambium cell is its repeated division in a tangential direction, quite contrary to Errera's Law. This is one of the main grounds on which Berthold concluded that Errera's Law, or the application of Plateaus's principle, derived from a study of soap films, could not be applied to dividing cells (1886). But there is another way of looking at the problem. Errera's Law should apply to a cell in equilibrium with its external environment. If the environment in any way establishes a gradient at a point on which the meristematic cell maintains itself, there would be good reason for anticipating a departure from Errera's Law and a formation of the plane of division in accordance with the gradient. But from an entirely different standpoint the significance of such a gradient in environment to the functioning of the cambial cell has already been suggested (Pearsall and Priestley, 1923). This gradient of hydrogen ion concentration, between the differentiating xylem, with sap relatively acid in reaction, and the differentiating phloem, relatively alkaline, is supported by many experimental observations and it has the further significance that at a certain range of hydrion concentration it is understandable that the plant proteins of the meristematic cell would have (1) minimum tendency to swell in water, (2) minimal electric charge, (3) least tendency to combine with inorganic salts forming charged protein ions, and possibly (4) (*e.g.* various papers of Vlès, 1925, whose work is summarised by Reiss, 1926) maximum tendency to combine with one another; these are the main characteristics of protein behaviour involved in protoplasmic synthesis and exhibited in the meristematic cell.

Naturally then the characteristic cambial divisions appear almost contemporaneously with the differentiation of xylem and phloem in the procambial ring, or a little in advance of visible signs of this differentiation.

In the Monocotyledon, where this cambial division does not occur, the procambial ring early breaks up into procambial strands and separate vascular bundles, associated with the development proceeding in the leaf initials. The absence of cambium in the Monocotyledon can only be the subject of speculation as yet, but a natural line of investigation opened up by the preceding argument, is that in the Monocotyledon the sap released by the differentiating xylem and phloem may fail to bracket the necessary range of hydrion concentration.

Behind the Root Apex. The Pericycle and Root Initials.

From this study of the factors shaping both meristematic cells and cell activity behind the shoot apex, it should be possible to turn to the root apex and subject the data there to similar analysis. Unfortunately, however, the activities of the deeply sunk apical pad of meristem and the zones of meristem lying behind it are less readily interpreted.

In the apical pad itself, in some root apices at least, as in *Vicia Faba*, and probably on the inner side of the apical pad in all cases, meristematic cell division nearly always produces walls in the direction at right angles to the root axis. But

the meristematic cells themselves are often not elongated in the direction of the axis, so that these divisions show no relation to Errera's Law. The possibility of a gradient which determines the maintained activity of the meristematic cell is thus suggested and is supported by many observations. The general reaction of the sap exuding from a cut root tip is definitely acid (Greenwood, and Pearsall, 1926), on the other hand, the reaction in the walls of the root cap will be that of the soil, and generally not far from pH 6-7. Most root growing points, furthermore, cease growth when the outside soil reaction becomes too acid (usually in the neighbourhood of pH 4·5). There is, therefore, a possibility that the root meristematic apex is thus maintained at a point on a gradient of hydrogen ion concentration which is relatively acid within, and less acid or neutral without. Behind the apex
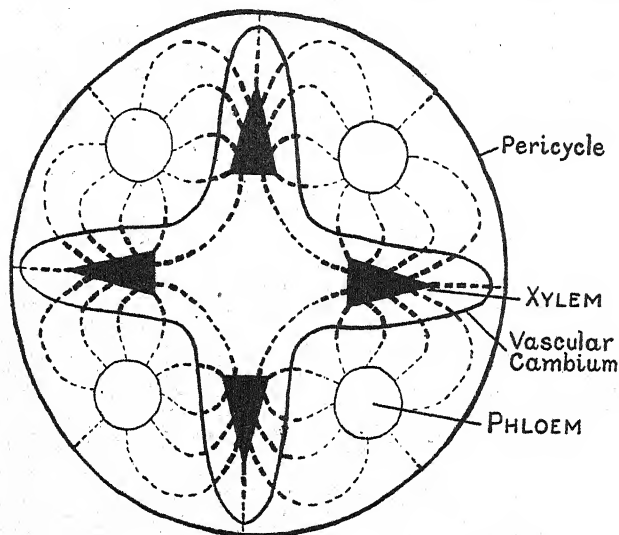
Fig. 7. Diagrammatic representation of radial arrangement of xylem and phloem in transverse section of the root. The gradient of hydrion concentration between acid xylem (black) and relatively alkaline phloem, is indicated by the density of the dotted lines. It will be seen that vascular cambium and pericycle may then arise at first in regions with a common intermediate hydrion concentration, and that the vascular cambium, when continuous, will separate an inner, more acid, zone from an outer, more alkaline zone.

the xylem and phloem differentiate not on the same radius, but on different radii. The result must be a very different position of the persistent intercalary meristem, if that arises from the maintenance of pH at a range somewhat intermediate between that existing in xylem and in phloem. Consideration of this problem (Fig. 7) will show that one probable position for such a maintained and persistent meristem is at a certain distance outside the whole series of xylem and phloem strands, and thus the future pericycle and endodermis maintain themselves meristematic for some time. Obviously also, between the xylem and phloem as before, a permanent gradient will be established and so later the cambium arises in this position.

If new root initials arise, they inevitably arise within the endodermis for reasons that have been stated fully elsewhere (Priestley and Pearsall, 1922).

Meristem cells require ample nourishment and the sap with organic solutes is retained within the endodermis. The meristematic layer within the endodermis is the pericycle and if a new root initial has to maintain itself between a relatively acid internal and an alkaline external gradient, the proper place for its appearance is in the pericycle opposite the xylem.

. If the Monocotyledon system fails to maintain a cambium between the xylem and phloem, it may also fail to maintain it for long at the periphery of the root stele. Some Monocotyledon roots, as the roots of Hyacinth, utterly fail to produce branch roots, and examination shows that in such roots vacuolated cells are found in the pericyclic region immediately behind the root apex.

When the vascular cambial layer has become active this lies across its usual gradient, relatively acid within and alkaline without, and after its formation as a continuous ring, future branch roots, either from stem or root, arise from this cambium with very few exceptions (Priestley, 1926 b). It is perhaps worth noting that the vascular cambium which is distinguished by more rapid cell division on its inner or more acid surface—the new cells added to the xylem always being more numerous than those added to the phloem—gives rise to a root apex, with acid reaction on its inner or stelar surface in which the apical meristem divides more rapidly on the inner surface and adds more cells to the stele than to the root cap.

The Phellogen.

On the other hand, on the outside of the root stele there is now, facing the pericycle from within, a continuous layer of relatively alkaline phloem. No more roots now arise from the pericycle, but instead a phellogen usually appears in it, and also appears near the periphery of the stem. This phellogen may be regarded as existing between an alkaline phloem on the inside and a relatively acid outer layer, formed by the release of fatty acids from the cells differentiating from the phellogen.

Even in the stem the phellogen may be regarded as facing the alkaline phloem, because although a parenchymatous cortex may intervene, the cortex has no characteristic hydrion concentrations to influence adjacent layers.

This point is of considerable importance. In any parenchyma cell, the sap inside the cell is retained by the semi-permeable protoplast. This layer retains all organic and most inorganic solutes completely (Hoagland and Davis, 1922–1926), and the pH of the internal sap is very steady, being buffered very adequately by both these salts and the protein in sap and protoplasm. In the wall, bathing the external surface of the protoplast there is also an aqueous medium which is practically unbuffered, salts moving into the protoplasts and not staying in this external sap. Its reaction is therefore easily altered, even by the CO_2 production of the cell. It is suggested, therefore, that in the walls of the cortical cells facing the phellogen, the pH of this external sap which bathes the phellogen cells, is determined rather by the phloem within, from which the basic contents diffuse out, than by the sap in the cortical protoplasts intervening which, though they have a ·

definite internal pH, are restrained by their efficient semi-permeable membranes from modifying the pH of the sap moving along their walls.

In shoot and root in adult Dicotyledon two intercalary meristems are found, therefore—the vascular cambium within, lying between internal relatively acid xylem and external alkaline phloem, and the cork phellogen without lying between internal relatively alkaline phloem and external cork cells releasing fatty acids.

Vegetative Propagation.

In cases of vegetative propagation from an axis containing two such intercalary meristems, the roots arise from the neighbourhood of the vascular cambium.
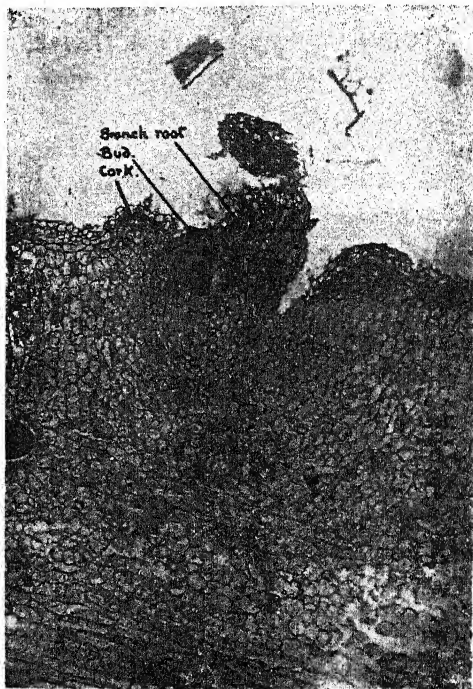
Fig. 8. Photograph of part of transverse section of a root of *Isatis tinctoria*, showing the endogenous origin of a bud to be associated both with the cork phellogen and the position of a branch root.

It is very suggestive furthermore that new shoots in many cases can be seen to arise from meristematic cells associated with the phellogen. This leads to a tentative suggestion as to a problem left unanswered earlier (p. 14). The procambial ring persisting below the superficial meristem of the shoot was left unexplained, as indeed also the maintenance of the activity of the superficial meristem itself. The cambial ring into which the procambial ring develops, is maintained across a gradient of hydrion concentration. But this suitable range of hydrion concentration for meristematic activity will persist beyond the ends of the differ-

entiated xylem and phloem. The procambial ring occupies the region where it will persist, and further away from too acid xylem and too alkaline phloem the gradient will reach a point all over the surface which is compatible with meristematic activity. Thus a region is obtained where the whole superficial surface is maintained in meristematic activity, but on its inner side, as the vascular elements differentiate forward, cells on the inside of the meristem pass into a gradient of hydrion concentration where vacuolation must ensue.

The picture that is then obtained of the relation of the superficial meristem of the shoot to the ends of the vascular elements, is closely analogous to that of the pericyclic meristem surrounding the strands of xylem and phloem in the root. It is, therefore, all the more suggestive that from such a pericycle may arise not only root initials as described above, but in certain plants (Beijerinck, 1886) new shoot initials may also spring endogenously from this pericycle. If the cork phellogen has previously developed in the pericycle, then the new shoot initials are associated with the phellogen, frequently in the region surrounding the base of branch roots (Priestley, 1926 *b*).

The above analysis of the conditions which govern meristematic activity is, of necessity, to a large extent speculative. There is however a certain amount of experimental evidence to support it, and with its help otherwise disconnected phenomena fall into place.

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FEEDING MECHANISMS IN THE INVERTEBRATES[1]

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(Received September 30, 1927.)

(With Twenty-five Text-figures.)

CONTENTS.

INTRODUCTION.

A SOUND knowledge of the structure and function of the feeding organs is of the first importance in the study of the living animal. Food is the first necessity of life and a knowledge of the means whereby it is obtained is essential if we are to estimate correctly the relationships of any animal, or group of animals, to their surroundings and to other animals.

Any attempt at a classification of feeding mechanisms must, perforce, be largely arbitrary, but this does not necessarily detract from its value as a convenient aid to knowledge. Feeding mechanisms develop in correlation with the environment and the available food and any classification of them must cut clean across the subdivisions of the animal kingdom. Animals of several different phyla which live on similar food possess feeding mechanisms of essentially the same nature, a fact which is best exemplified by the widespread occurrence of ciliary feeding mechanisms, while within a single class, such as the Gastropoda, there may be found examples of almost every type of feeding mechanism. It is necessary, therefore, that feeding mechanisms should be considered in relation to the food and environment, and not to the systematic position, of the animals which possess them.

There are well-defined correlations between (1) the habitat and available food of any animal, (2) the type of feeding mechanism, (3) the structure of the alimentary canal, and (4) the nature of the digestive processes and the relative strength of the various enzymes. Thus it is possible from a study of, say, any two of these to forecast with some degree of accuracy the nature of the other two. From a laboratory study of the feeding mechanisms and digestive processes of an animal

we can form a fairly accurate idea of its habitat and food. Although this paper will deal exclusively with feeding mechanisms, I hope it may later be possible to discuss the structure and function of the alimentary system from a similar standpoint.

It is clearly impossible in so short a space to give a complete account of feeding mechanisms in the Invertebrates. A general classification with appropriate examples of each type taken from the most recent work on the subject is all that can be accomplished. Most of the feeding mechanisms described are those of marine Invertebrates, those of the Protozoa and the Insecta, in particular, are not treated in detail, and for a full account of these reference must be made to appropriate works on Protozoology and Entomology (*e.g.* those of Calkins (1926) and Sharp (1895, 1899) respectively). The attempt has been made to collect, in the bibliography, references to all the most important papers, but, owing to the extremely scattered nature of the literature, it is inevitable that some have been overlooked.

PREVIOUS CLASSIFICATIONS.

The principal attempts at the classification of feeding mechanisms are those of Jordan (1913), Blegvad (1914), Hirsch (1915), Hunt (1925) and finally the recently published work of Jordan and Hirsch (1927). A brief examination of these is a necessary preliminary to the description of the classification adopted in this paper.

Jordan (1913) divides up animals according to the nature of their feeding mechanisms in the following way:

1. Whirlers (Strudler).
E.g. all ciliary feeders, and others which feed on small particles.
2. Snarers (Schlinger).
(*a*) Those which swallow whole objects of prey, *e.g.* Coelenterates.
(*b*) Those which prepare the food by mastication, *e.g.* some Arthropods.
(*c*) Those which possess a muscular pharynx, *e.g.* Turbellarians and Oligochaetes.
(*d*) Molluscs provided with a radula.
(*e*) Those which digest the food external to the gut, *e.g.* Cephalopods, some Insects.
3. Suckers (Sauger).
E.g. all which feed by sucking in fluid matter.

The above classification covers the entire Invertebrata, and, with regard to the three main divisions, has been largely followed in this paper. It does not, however, go into any great detail, and each of the main divisions requires subdivision.

Blegvad (1914) has also divided animals into groups according to the arrangement of their feeding organs. His classification is more detailed as shown below:

A. Animals without hard prehensile or masticatory organs.
I. Those feeding entirely by means of pseudopodia, *e.g.* Foraminifera.
II. Those feeding by means of a ciliate epithelium occasioning a constant current of water through a portion of their body, *e.g.*:
(*a*) Without gills, *e.g.* Porifera.
(*b*) With gills, *e.g.* Lamellibranchia, Ascidiae.

III. Those feeding by means of prehensile arms or tentacles without nematocysts but frequently furnished with cilia, *e.g.* many Polychaetes and Holothurians.

IV. Those feeding by means of a soft, generally extroversible gullet, or proboscis, for drawing up the detritus of the bottom, *e.g.* many Polychaetes, and Gephyrea.

V. Those feeding by means of ambulacral feet, *e.g. Echinoidea atelostomata* and *Amphiura* spp., many Ophiuroids, Asteroids.

VI. Those feeding by means of prehensile tentacles furnished with nematocysts, *i.e.* Coelenterates.

B. Animals with hard prehensile or masticatory organs.

I. Those feeding by means of a radula, *i.e.* Gastropods.

II. Those feeding by means of an extroversible gullet, furnished with hard mandibles or teeth, *e.g.* various Polychaetes, Priapulidae.

III. Those feeding by means of setae-covered limbs with chitinous cuticle, generally also having a masticatory stomach furnished with chitinous plates, *e.g.* Crustacea, Dipterous larvae.

IV. Those feeding by means of a masticatory organ with five calcareous teeth, *e.g.* Echinoidea.

Blegvad only studied animals from the sea bottom and even for this limited number his classification is artificial and unsatisfactory. The division of animals into two large groups according to the presence or absence of hard prehensile or masticatory organs is unsound, resulting, for example, in ciliary feeders and carnivorous Coelenterates being placed in the same division, and animals which feed on fine particles being placed in different divisions according to whether they do so by means of cilia or setous limbs.

Bottom-living animals are also divided up by Blegvad according to the nature of their food[1] in the following way:

1. Herbivores.
2. Carnivores.
3. Detritus-eaters.
(*a*) Herbivorous detritus-eaters.
(*b*) Carnivorous detritus-eaters.

Hunt (1925) has shown, however, that this classification also is in many respects unsatisfactory.

Hirsch (1915) in an important paper on feeding and digestion in Gastropods

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[1] Rauschenplat (1901) classified marine animals according to their type of food thus:
1. Feeders on large plants.
2. Feeders on small plants.
3. Feeders on animals.
4. Feeders on plankton.
5. Feeders on detritus.
Brand (1927), discussing the food of marine Polychaetes and other worms, divides them into four groups, 1, 3, and 5 of Rauschenplat's classification together with Feeders on small organisms and detritus.

divides these animals into four groups according to the nature of their feeding mechanisms, as shown below:

1. Whirlers (Strudler), *e.g.* Thecosomatous Pteropods, *Limnaea*.

2. Snarers (Schlinger), *e.g. Pleurobranchaea, Pterotrachea*.

3. Scrapers (Kratzer), *e.g. Murex, Natica, Tritonium*.

4. Suckers and Parasites (Sauger), *e.g. Hermaea, Neomenia*, etc. and ecto-parasites.

This classification, though it deals only with Gastropods, is of value because of the variety of feeding mechanisms found in this Class. It follows Jordan's classification very closely but with the useful addition of a fourth division of Scrapers.

In his work on the food of the bottom fauna at Plymouth, Hunt (1925) classified the bottom-living animals largely according to the type of food, in the following manner:

A. Carnivores. Animals which feed mainly upon other animals, either living or as carrion.

B. Suspension-feeders. Animals which feed by selecting from the surrounding water the suspended micro-organisms and detritus.

C. Deposit-feeders. Animals which feed upon the detritus deposited on the bottom, together with its associated micro-organisms.

This classification avoids the errors of Blegvad's, and it fits in closely with the distribution of the different types of feeding mechanisms, showing the correlation between the food and feeding mechanisms. It is, of course, only concerned with a section of the Invertebrates.

Jordan and Hirsch (1927) have recently given the most detailed general account of feeding mechanisms as yet published. The classification which they adopt is different from that of their earlier works, being as follows:

A. Animals which do not break up large food masses (Mikrophage Tiere).

1. True particle feeders.

(*a*) Filterers, *e.g.* Lamellibranchs, Porifera, lower Crustacea, etc.

(*b*) Mucus entanglers, *e.g. Vermetus*.

(*c*) Those with tasting appendages, *e.g. Dentalium*, Cucumarians, etc.

2. Suckers, *e.g.* blood sucking Diptera, Acarines, parasitic worms, etc.

B. Animals which can break up large food masses (Die makrophagen Tiere (Zerkleinerer)).

3. Snarers, *e.g.* Coelenterates, Asteroids, *Sagitta*, etc. etc.

4. Masticators (Kauer), most Vertebrates, Rotifers, Decapod Crustacea (latter two mastication in stomach).

5. Scrapers, *e.g.* Echinoids, many Snails, Teredinidae, Cephalopods, many Insecta, etc. etc.

6. External Digestors (Aussenverdauer), *e.g.* larvae of *Carabus, Dytiscus*, Muscidae, etc.

Under neither A nor B come:

7. Animals without alimentary systems (Parenteralen), *e.g.* many endoparasites.

Although this classification is extremely comprehensive, including both Verte-brates and Invertebrates (though not the Protozoa), and in many ways very valuable, for it is illustrated with a great wealth of examples, yet in its arrangement it represents rather a retrogression from the original classifications of its authors. It does not seem logical to group together under the same general heading the fine particle feeders and the suckers, nor to separate the ectoparasitic suckers so completely from the endoparasites without alimentary systems.

OUTLINE OF PROPOSED CLASSIFICATION.

The feeding mechanisms of Invertebrates are here divided up and described in the following manner:

I. MECHANISMS FOR DEALING WITH SMALL PARTICLES.
(*a*) Pseudopodial.
(*b*) Ciliary.
(*c*) Tentacular.
(*d*) Mucoid.
(*e*) Muscular.
(*f*) Setous.

II. MECHANISMS FOR DEALING WITH LARGE PARTICLES OR MASSES.
A. For Swallowing Inactive Food, *e.g.* bottom deposits, etc.
B. For Scraping and Boring.
C. For Seizing Prey.
 (i) For Seizing and Swallowing only.
 (ii) For Seizing and Masticating.
 (iii) For Seizing followed by External Digestion.

III. MECHANISMS FOR TAKING IN FLUID OR SOFT TISSUES.
 (i) For Piercing and Sucking.
 (ii) For Sucking only.
 (iii) For Absorption through Surface of Body.

GENERAL ACCOUNT OF FEEDING MECHANISMS.

A short account of the different types of feeding mechanisms illustrating the above classification will occupy the remainder of this paper. In each subdivision, for the sake of convenience, the different groups possessing that particular type of mechanism will be treated in the conventional order, *i.e.* beginning with the Protozoa.

I. MECHANISMS FOR DEALING WITH SMALL PARTICLES.

(*a*) *Pseudopodial.*

This type is found only in the Protozoa where it is possessed by the Foraminifera and Radiolaria. The process of feeding in the former has been described by Bütschli (1880) and Jensen (1901). The pseudopodia are long and root-like, branch-ing and anastomosing frequently, some are broad and others very fine but the

ultimate branches are always extremely tenuous. There is a continuous streaming of the protoplasm on either side of each pseudopodial filament, one current being centrifugal and the other centripetal, the length of the filament at any moment depending on the relative strengths of the two. Minute particles become entangled in this viscid protoplasmic network, pass under the influence of the centripetal current, either directly or after being first carried to the tip by the centrifugal current, and are carried towards the body. Jensen placed starch grains in the protoplasmic network of *Orbitolites complanatus* and observed that they began to be carried inwards after 2–10 minutes by a series of jerky movements, being finally ingested in the protoplasm of the body. The size of the particles ingested depends on the size of the pores through which the pseudopodia pass. In the Radiolaria (Haeckel, 1862) conditions are very similar, food being caught on the sticky pseudopodia. Later there is a streaming of protoplasm in that direction followed by a centripetal streaming on the protoplasm of the pseudopodium whereby the food is carried within the outer layer of the body. The food of both Foraminifera and Radiolaria consists largely of fine plankton.

(b) Ciliary.

The use of cilia as a means to catch fine food particles is widespread and types of ciliary feeding mechanisms, from the simple spirals of cilia found in the Protozoa to the elaborate mechanisms of the Lamellibranchs, are found in almost all phyla with the notable exception of the Arthropoda.

Protozoa. Many Ciliata obtain their food by means of cilia, especially members of the Holotrichida (*e.g. Paramecium*), Heterotrichida (*e.g. Stentor*), and Peritrichida (*e.g. Vorticella*), which, owing to the small and non-extensile mouth, are unable to feed on the relatively large particles which form the food of many Ciliates. The feeding mechanisms take the form of whorls or spirals of cilia. On one side of the body of *Paramecium* there is a broad, oblique groove, or peristome, which extends forward from the mouth which lies near the middle of the body. This groove is lined with oral cilia which, to quote Jennings (1915, p. 46), "cause a current of water to flow rapidly along the oral groove. In the water are the bacteria upon which *Paramecium* feeds; they are carried by this current directly to the mouth. In the gullet is a vibrating membrane which carries particles inward...." A feeding-cone is produced for a short distance in front of the animals while they are feeding, either at rest or, under certain circumstances (see Mast and Lashley, 1916), while in motion. Jennings (p. 171) has described the feeding of *Stentor*; the edge of the disc is lined with large peristomal cilia whose beating forms a vortex at the base of which lies the mouth into which particles descend from all sides. Greenwood (1894) has shown that in *Carchesium*, a genus closely allied to *Vorticella*, fine food particles are caught in a vortex produced by the cilia surrounding the peristome and passed into the mouth which leads into a slightly sinuous, ciliated pharynx which becomes narrower and finally merges into a small dilated oesophagus. There are many other modifications of the ciliary feeding mechanisms in the Ciliata, as outlined in Calkins (1926).

The Choanoflagellates collect fine particles from the water current by the lashing

of their flagella. According to Franzé (1893) food is not ingested within the collars, but in a receptive area some distance below the base of the collar.

Porifera. The food current in sponges is created, as in the Choanoflagellates, by the lashing of the flagella of the collar cells, the body of the sponge acting as a sieve. Details of the canal system are too well-known to need further description. Water enters the inhalent system by way of the ostia, passing through the prosopyles into the flagellated chambers and thence via the apopyles into the exhalent system, finally leaving the body through the osculum. Vosmaer and Pekelharing (1898) thought that the motion of the flagella was quite irregular, but van Trigt (1919), who worked on the Spongillidae, states that movement is quite regular, the motion, under normal conditions, being in a spiral or undulating line, "a very rapid succession of waves of small amplitude passing along the flagellum from the base to the top." The current of water follows the direction of the axis of the spiral formed by the movements of the flagellum. As shown in Fig. 1, the collar cells have a definite arrangement with relation to the prosopyle and apopyle, water and fine particles being drawn in through the former, food being ingested in the choanocytes a little distance below the base of the collars, and the filtered water passing out through the apopyle. Particles too large to pass through the prosopyle are ingested by the layer of "apparently undifferentiated" protoplasm which lies on the outer surface of the flagellated chambers. In the higher sponges the pinacocytes are also concerned with ingestion.
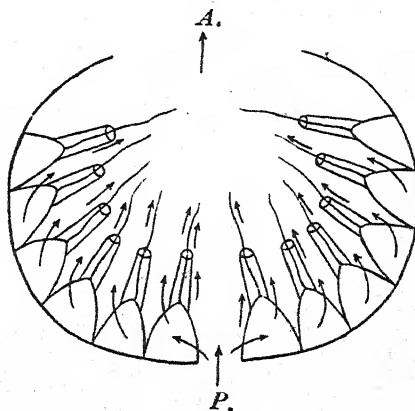
Fig. 1. Diagrammatic representation of the water current inside a flagellated chamber of Spongilla. *A*, apopyle; *P*, prosopyle. Arrows indicate the direction of the water currents, from which food particles are removed as they pass the base of the collars. (From van Trigt.)

Coelenterata. Cilia are usually present in the alimentary system of the coelenterates[1] but are seldom used for the capture of food. In the Actinarians, Carlgren (1905) states that the cilia covering the entire ectoderm of *Protanthea* carry fine particles to the mouth whence they are swept into the stomach by the cilia lining the stomadaeum, similar cilia are present in *Gonactinia* except in the proximal region of the body wall, while in *Halcampa, Sagartia* and *Metridium*, the tentacles, disc and stomadaeum alone are ciliated and work in conjunction. In the Madreporarian *Caryophyllia*, where the tentacles have nematocysts, particles are carried into the stomach by cilia lining the inner part of the disc and the stomadaeum. Parker (1896, 1905) considers that the movement of the cilia on the lips of *Metridium* is reversible under certain conditions, although the cilia on the tentacles always beat outwards. According to Elmhirst (1925) there are separate sets of cilia in the stomadaeum, those on the ridges beating outwards and those in the grooves

[1] For details regarding internal ciliation see Widmark (1911) for *Aurelia* and *Cyanea*, and Gemmill (1918) for *Pleurobrachia*, (1919) for *Melicertidium* and (1920) for the ephyrea of *Aurelia*.

inwards, and that material is passed in or out according to the state of contraction of the stomadaeum. Duerden (1906) states that *Fungia* and *Favia* have the disc covered with cilia and mucus; normally the cilia beat outwards but nutritive substances placed on the disc cause an increased secretion of mucus in which particles are entangled and drawn inwards, as Duerden thinks, by a reversal of ciliary movement. In the Scyphozoa, Orton (1922) has shown that plankton is collected by both ex- and sub-umbrella surfaces of *Aurelia aurita*, being carried to the edges where it collects in masses midway between the tentaculocysts before being "licked off" by the oral arms, the cilia of which convey it into the gastric pouches.

Echinodermata. A most valuable account of ciliary mechanisms in Echinoderms has been given by Gislén (1924). With the doubtful exception of the planktonic *Pelagothuria*, the Holothuroidea do not possess external ciliation. With a few possible exceptions, where it may assist in feeding, the ciliation of both the Echinoidea and Ophiuroidea is concerned largely with respiration and cleansing: Gemmill (1915) demonstrated the presence, in Porania, of powerful ciliary currents leading to the mouth and showed that it can feed in this manner for long periods. He found weaker feeding currents in *Astropecten* and still slighter ones in *Solaster*. Gislén confirms this and has shown that the other Asteroids, *Pseudarchaster*, *Luidia*, *Pontaster*, *Ctenodiscus* and *Poraniomorpha* also possess ciliary feeding currents. In the Crinoids ciliary feeding mechanisms are universal. The ambulacral groove is lined with cilia which beat towards the mouth and also possesses many mucus glands. The pinnules are covered with small tentacles, unciliated but with mucus glands, which jerk particles into the ambulacral groove. Normally this is shut but during feeding it opens and food is carried along it to the small mouth which opens to receive it. In the Comasterids, where ciliation is slighter, Gislén thinks that the combs assist in the collection of food. The stomach contents of Crinoids consist of plankton and detritus.

Polychaeta. The cryptocephalous polychaetes feed on suspended matter which they collect by means of the cilia and mucus on their gills. On the pinnules of the tentacles of such genera as *Spirorbis*, *Pomatoceros*, *Hydroides*, *Branchiomma*, *Sabella* and *Filograna* there are, according to Orton (1914), lateral current-producing cilia and also frontal cilia for the collection and transportation of food, which is carried to the axes of the tentacles and thence to the mouth. The feeding of *Chaetopterus* has been described by Joyeux-Laffuie (1890). This animal lives in a parchment-like tube and draws in suspended matter by ciliary action, the larger particles falling directly into the wide buccal funnel and the smaller ones being caught in ciliary currents within the dorsal grooves on the large lateral extensions of the twelfth segment which lead into a median dorsal groove which extends to the posterior margin of the buccal funnel. There the food accumulates as small masses which finally fall inwards towards the mouth. Selection is less rigorous in *Chaetopterus*, and Hunt (1925) has shown that the stomach contents are less finely sorted than in the other tube-dwelling worms.

Gephyrea. Some Echiuroidea probably feed by means of the ciliary current within the ventral groove of the proboscis, the edges of which can be approximated to

form a tube. The great extensibility of the proboscis in *Bonellia* may aid the animal in its search for food.

Rotifera. The majority of the Rotifers collect food with the ciliary currents on the disc and in the pharynx, and a detailed account of the morphology of these organs has been given by de Beauchamp (1907). According to Naumann (1923), fine particles are carried into the pharynx by the action of disc cilia which produce a current from before backwards, unsuitable material being rejected from the pharynx in an outgoing current. The discs do not work continuously but the entrance of food into the gut is independent of them. Rezvoj (1926) states that the size of the particles swallowed varies according to the size of the opening into the mastax in which food is triturated, and so selection is largely quantitative. Excess material is removed in a ventral, outgoing tract of cilia. Naumann (1924) emphasises the resemblance between the feeding of Rotifers and of certain Ciliates, while Mast and Lashley (*loc. cit.*) have noted the presence in both of these groups of a feeding-cone.

Polyzoa. Ciliary feeding is universal amongst these animals, whose mouths are fringed with a ring of ciliated tentacles, the currents on the two sides of which run in opposite directions (Gilchrist, 1908, p. 163), by means of which fine plankton and other minute particles (such as have been identified in the stomachs of *Lepralia* and *Cellaria* by Hunt (p. 574)) are carried into the alimentary canal. It is possible, though never definitely proved, that the avicularia present in some genera and which certainly capture small animals hold these fast until they disintegrate when the resultant particles are swept into the mouth by the ciliary currents.

Phoronidae. In *Phoronis capensis* Gilchrist (1908) describes the presence of an inhalent current which passes in the direction of the mouth between the outer and inner circle of tentacles on the lophophore, and exhalent currents flowing out between the expanded tentacles, downwards between those of the outer row and upwards between those of the inner row. The tentacles are ciliated, the cilia on the two sides beating in opposite directions, towards and away from the mouth. Particles are caught by the former and carried towards the mouth, there to be swallowed or rejected when they are carried by the distally beating cilia to the tip of the tentacles where they are dropped off, ciliary action ceasing during the latter operation.

Brachiopoda. Shipley (1883), working on *Argiope*, appears to have been the first to state definitely that the lophophores of Brachiopods are ciliary feeding mechanisms. Orton (1914) has given a detailed account of the feeding mechanisms of *Crania* and *Terebratula*. The mantle chamber is divided into two physiologically distinct compartments by the two lophophores which work independently. A strong inhalent current is produced on either side of the mantle chamber, while a single exhalent current passes out between and above them. The former are produced by the beating of cilia on the sides of the filaments which fringe the lophophores, on the mantle, and on the body of the lophophores. As shown diagrammatically in Fig. 2, the inhalent current is drawn in beneath the lowest, and widest, whorl of the lophophore.

The heaviest particles in suspension drop on to the lower mantle surface, as

shown by the dotted arrows near *A*, from which they are rejected by ciliary action, but water and the finer particles are drawn upwards through the superposed whorls of the lophophore, which is everywhere fringed with a double row of filaments which alternate with one another to form a series of sieves. On the lower or frontal surface of these are cilia which beat towards the base of the filaments (see Fig.
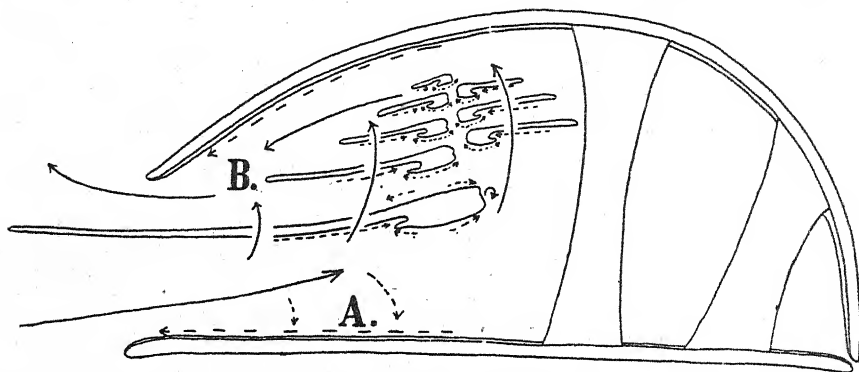
Fig. 2. Diagram of the currents present in the mantle cavity of a Brachiopod as represented by *Crania*. *A*, Inhalent chamber of one side; *B*, exhalent chamber. Large arrows indicate course of main currents, inhalent below, exhalent above; between currents through the lophophore. The dotted arrows on the lophophore and gill-filaments indicate course of the food-collecting streams. (After Orton (1914), Fig. 4. By kind permission of the Marine Biological Association.)
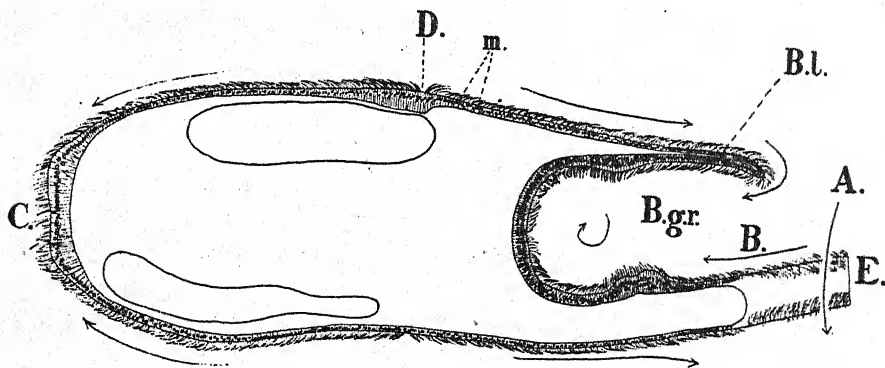
Fig. 3. Semi-diagrammatic transverse section of the lophophore of *Crania*, arrows indicating directions of currents. *A*, arrow showing direction of current produced by lateral cilia on filament; *B*, direction of current produced by frontal cilia on filaments; *B.gr.* buccal groove; *B.l.* buccal lip; *C*, inner edge of lophophore along which particles carried to its base; *D*, point dividing regions of differently beating cilia; *E*, base of gill-filament arising from lophophore; *m.* mucus. (After Orton (1914), Fig. 6. By kind permission of the Marine Biological Association.)[1]

3, *B*.), a copious supply of mucus is secreted and in it particles are entangled before being conveyed to the buccal groove (*B.gr.*) which borders the outer edge of the lophophore. On the sides of the filaments are the lateral cilia which beat upwards (*A*) and create the water current. On the lophophore itself, as shown in Fig. 3, the cilia on the sides nearest the filaments carry particles in this direction or into the

[1] In this figure the *ventral* surface of the lophophore is uppermost.

buccal groove, while those on the further sides beat in the opposite direction and assist in the rejection of particles. As shown in the figures, the filaments are attached to the upper side of the buccal groove, the lower side of which is bounded by a protruding buccal lip (*B.l.*). Within this groove is a powerful current which carries food towards the mouth above which the two lophophores unite. After passing through the whorls of the lophophore, the water currents from either side unite with one another and with currents produced by the mantle cilia, to form the median, dorsal exhalent current. Similar conditions have been observed by the writer in *Waldheimia*, though it appeared that the ciliary action of the filaments was often assisted by muscular movements.

Mollusca. In the Lamellibranchs ciliary feeding mechanisms attain their highest development, but they also occur in several groups of the Gastropoda and these have especial interest because each has developed independently.

Gastropoda. Ciliary mechanisms are present in certain of the sedentary Pectinibranchia and the conditions in one of these, *Crepidula fornicata*, has been investigated in detail by Orton (1912). The mantle cavity lies dorsal to the "head"
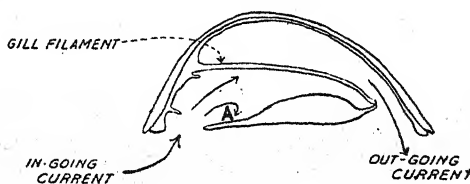
Fig. 4. Diagram of the spacial relations in the mantle cavity of *Crepidula* in transverse section. *A*, position of forwardly directed stream. (After Orton (1912), Fig. 7. By kind permission of the Marine Biological Association.)

and is divided into a left ventro-lateral, and a right dorso-lateral, chamber by the gill which consists of a row of some four hundred free filaments (see Fig. 5, *g.f.*). These bear cilia which create an inhalent current which enters the ventral chamber and an exhalent current which passes out from the dorsal chamber, as represented in Fig. 4. The gill filaments are attached to the left side of the mantle cavity, they are flattened antero-posteriorly and stand a little distance apart except at the tips where they are flattened dorso-ventrally and form a continuous membrane. On their sides are lateral cilia which beat upwards and cause the water current, while on the ventral and dorsal surfaces respectively are frontal and abfrontal cilia which beat towards the tips. After entering the wide mantle cavity, the velocity of the inhalent current decreases and the heavier particles tend to fall (see *A*, Fig. 4) and be caught in forwardly directed currents in the left of the mantle cavity which carry them into a food-pouch (*f.p.*), consisting of a groove within semicircular folds, immediately below the mouth. Here they are worked up into pellets with mucus which may be eaten but are more often rejected, being either carried to the edge of the shell by cilia or else pushed into the exhalent current. The fine particles which remain in suspension are collected by the gills, which are covered with mucus secreted by an endostyle (*EN.*) at their base (Orton, 1914), and then carried across

the ventral surface by the frontal cilia. The collected particles are swept by the cilia on the ventral and dorsal surfaces of the filaments into the food channel (Fig. 5, *f.ch.*) which runs along the left side of the mantle cavity, and consists of a ciliated groove practically roofed in by the flattened tips of the filaments (which
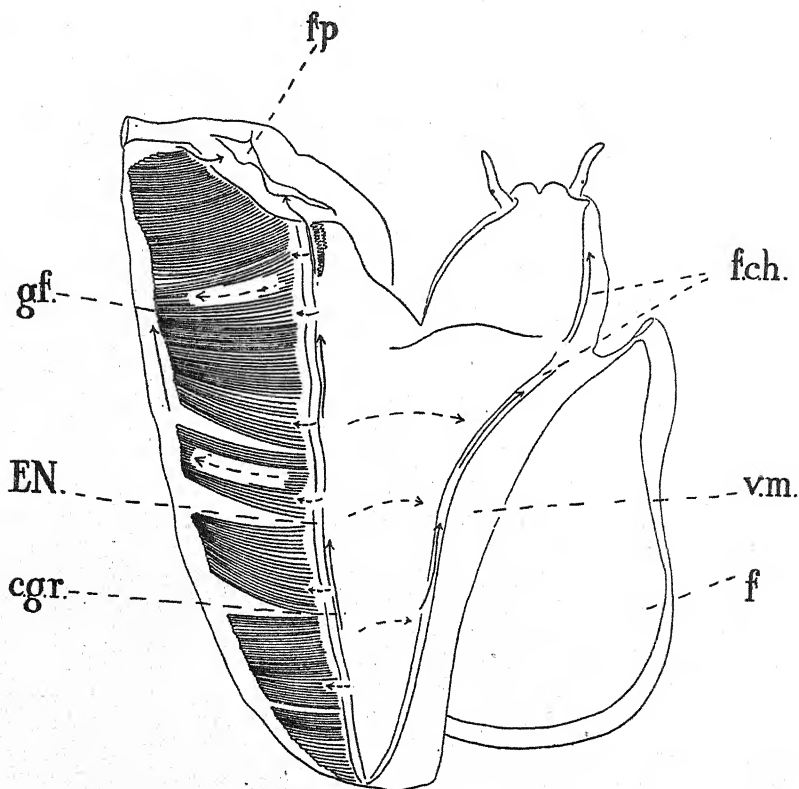
Fig. 5. Mantle cavity and gill of *Crepidula*, showing endostyle. The animal has been taken from its shell and the mantle turned over to the left. *EN.* endostyle; *c.gr.* ciliated groove on left of endostyle carrying mucus to food-pouch; *f.p.* food-pouch; *f.ch.* food-channel; *f.* foot; *v.m.* visceral mass; *g.f.* gill filaments. Dotted arrows on endostyle indicate directions in which mucus is lashed from endostyle on to the base of the gill-filaments. (After Orton (1914), Fig. 10. By kind permission of the Marine Biological Association.)

are shown turned back in Fig. 5). In it mucus-laden strings are carried forwards to be seized by the radula which conveys them into the buccal mass where they are retained by the small mandibles before being swallowed. The feeding of *Calyptraea* is identical with, and that of *Capulus* very similar to, that described for *Crepidula* and Orton thinks there is good reason for suspecting that all sedentary Pectinibranchia feed in some such manner.

The shelled or Thecosomatous Pteropods feed by means of ciliary currents present on the unpaired middle lobe and the two paired side lobes of the foot. The feeding mechanisms of *Cavolinia*, *Creseis*, *Cymbulia* and *Gleba* have been studied by Yonge (1926 *b*). In the two first food is collected on a wide ciliated

field (Fig. 6, *c.f.*) at the posterior side of the wings (*w.*). Mucus is secreted in this region and entangled food is carried by cilia to the mouth (*m.*) which is enclosed in a triangle of folds (*m.l.*, *s.l.*) representing, as Meisenheimer (1905) has shown, the lobes of the foot, the ciliated fields being extensions of the side lobes. The cilia on the folds carry particles to the mouth; but surplus matter may be rejected either by a contraction and consequent meeting of the folds or by an outgoing tract of cilia (*o.t.*) lying between the side lobes anterior to the mouth. In *Creseis* the ciliated field is more localised. In *Cymbulia* there are no ciliated fields, cilia being confined
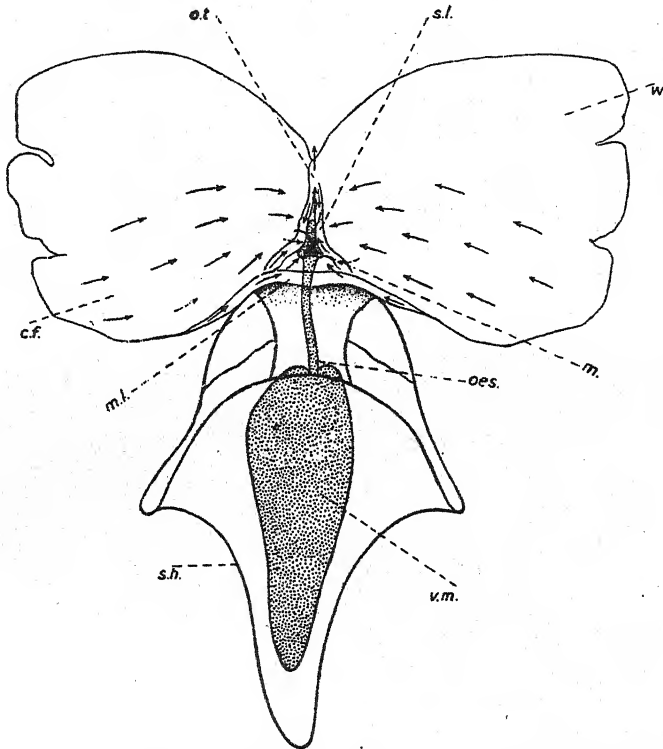
Fig. 6. *Cavolinia inflexa*, whole animal from ventral aspect showing ciliary currents. ×7. *c.f.* ciliated field; *m.* mouth; *m.l.* middle lobe of foot; *o.t.* outgoing tract of cilia; *oes.* oesophagus; *s.l.* side lobe of foot; *sh.* shell; *v.m.* visceral mass; *w.* wing. (After Yonge (1926 *b*), Fig. 1. By kind permission of the Linnean Society.)

to a pair of lateral grooves which represent the lobes of the foot and which meet above the mouth. Their cilia carry food to the mouth while there is an outgoing tract anterior to the mouth. In *Gleba* (Fig. 7) the reduction in the ciliary mechanism has been taken a stage further, the mouth (*m.*) lying at the end of a proboscis (*p.*) up the sides of which run ciliated tracts (*t.s.*) which convey particles into the grooves formed from the lobes of the foot (*m.l.*, *s.l.*) which are confined to the tip of the proboscis. The outgoing tract lies in the middle line beneath the proboscis, *i.e.* in the same relative position as in the other genera. Correlated with the increased specialisation and efficiency of the ciliary mechanisms, there is a reduction in the

buccal mass and its associated structures, radula, jaws and "salivary" glands (handed down from carnivorous ancestors), which, though comparatively well developed in *Cavolinia* and *Creseis*, are vestigial in *Cymbulia* and absent in *Gleba*.

Some freshwater Pulmonates feed, at any rate partially, with the aid of cilia. Thus Brockmeier (1898) has shown that *Limnaea* stops now and again while creeping suspended from the surface film and lets down the anterior end of its foot. Fine organisms are caught in the mucus and carried by the cilia on the
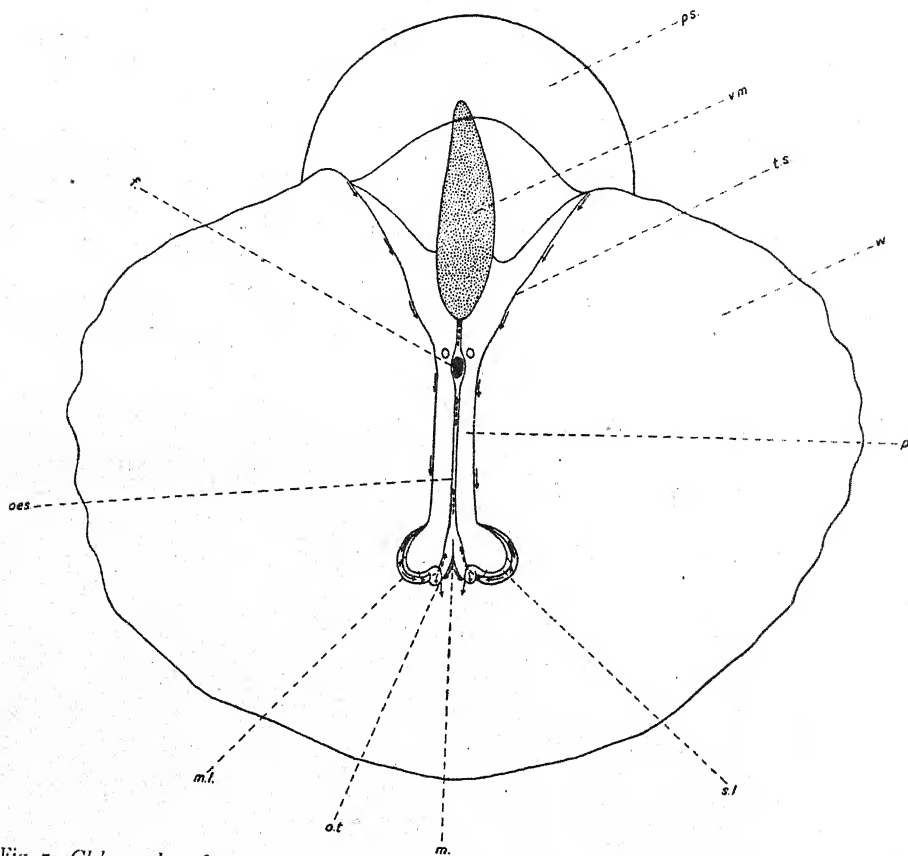
Fig. 7. *Gleba cordata*, from oral aspect showing ciliary currents. ×2½. *f.* food in oesophagus; *p.* proboscis; *p.s.* pseudoconch; *t.s.* ciliated tract on side of proboscis. Other lettering as in Fig. 6. (After Yonge (1926 *b*), Fig. 4. By kind permission of the Linnean Society.)

creeping sole to the posterior end where they collect. At the end of each period of feeding the head is bent back and the food "licked off" before the animal moves to a new feeding place.

Lamellibranchia. With the exception of the Septibranchia and, to some extent, the Teredinidae, the Lamellibranchia all feed by means of ciliary currents on the gills and labial palps. The elaborate feeding mechanisms have been the subject of many investigations and an extensive literature has grown up on the

subject. A short account of the process of feeding in such a typical Lamellibranch as *Ostrea edulis* (Yonge, 1926 *a*) will be given before passing to the discussion of the ciliary currents on the gills, palps and other surfaces in the mantle cavity.

In many Lamellibranchs such as *Schizotherus* (Kellogg, 1915) or *Mya* (Yonge,
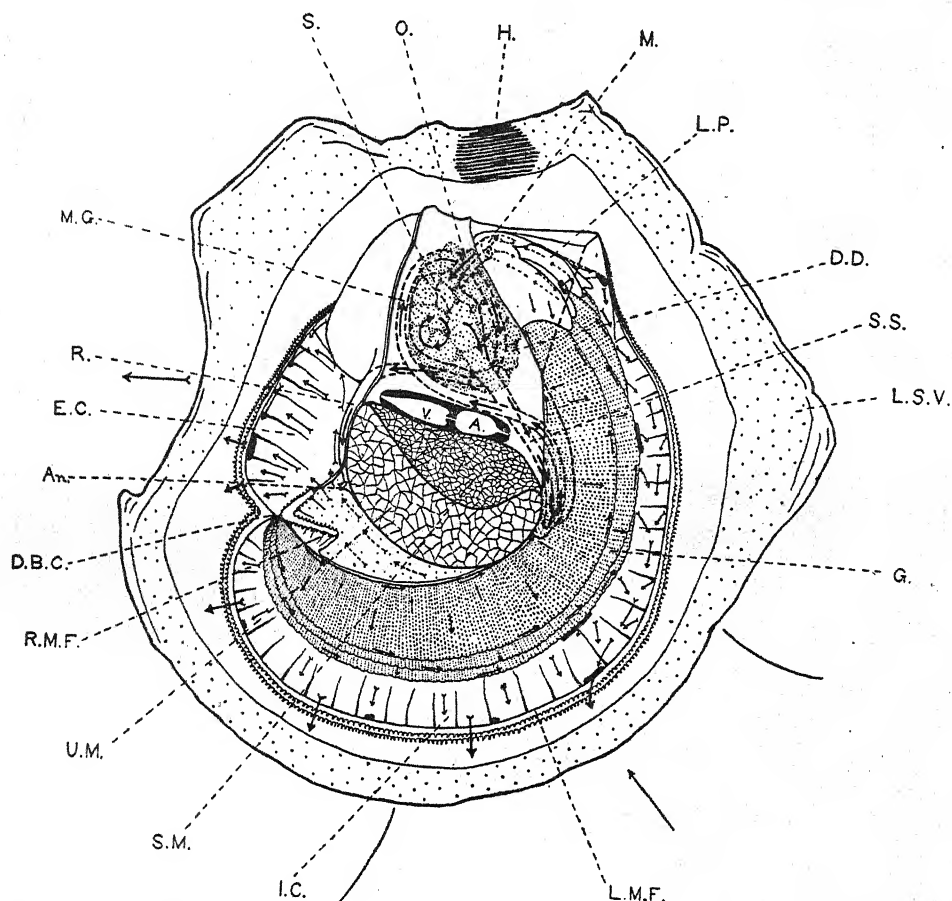
Fig. 8. *Ostrea edulis*, right shell valve and mantle fold removed. ×1. *An.* anus; *D.B.C.* division between inhalent and exhalent chambers; *D.D.* digestive diverticula; *E.C.* exhalent chamber; *G.* gills; *I.C.* inhalent chamber; *L.M.F.* left mantle fold; *L.P.* labial palps; *L.S.V.* left shell valve; *M.* mouth; *M.G.* mid-gut; *O.* oesophagus; *R.* rectum; *R.M.F.* right mantle fold; *S.* stomach; *S.S.* style-sac. Large arrows external to shell denote direction of ingoing and outgoing currents, within shell plain arrows denote ingoing currents and feathered arrows outgoing currents, broken arrows (except in gut) denote currents on under surfaces. (After Yonge (1926 *a*), Fig. 1. By kind permission of the Marine Biological Association.)

1923) the inhalent and exhalent currents enter and leave the body respectively by way of siphons at the posterior end of the body, the mantle folds being united for the greater part. In *Ostrea* there are no siphons and the mantle folds are united only at the junction of the inhalent and exhalent chambers (see Fig. 8, *D.B.C.*), but the inhalent current is restricted to a ventral area (between the thick curved

lines in the figure) owing to the opposition of the mantle folds. This current is drawn into the inhalent chamber (*I.C.*) and through the gills (*G.*), fine particles being left on the surface of the latter where they are entangled in mucus and carried either to the free margin of the lamellae or else to their base. In either case they are carried towards the mouth (*M.*) as indicated by the arrows. Two pairs of labial palps (*L.P.*) guard the mouth and sort the material passed on to them by the gills, rejecting the larger particles or masses and only allowing the smallest to reach the mouth. The water which passes through the gill forms the exhalent current which leaves the body posteriorly by way of the exhalent chamber (*E.C.*) as indicated by the large feathered arrow. Accounts of ciliary mechanisms in a great number of Lamellibranchs are given in the beautiful paper by Kellogg (1915) and also by Orton (1912, 1913) and others.

Gills.

These organs are entirely responsible for the production of the water current and also, except in the Protobranchs, for the collection of food. Their structure has
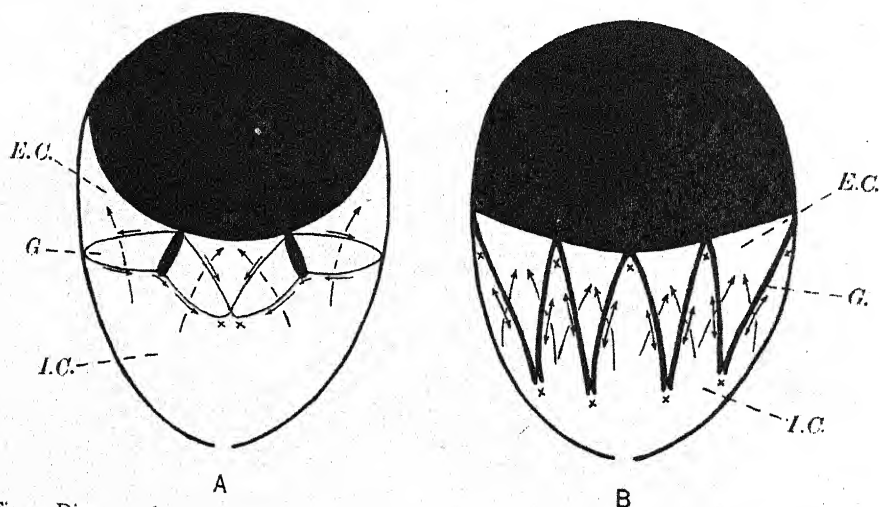
Fig. 9. Diagrams showing division of mantle cavity into inhalent (*I.C.*) and exhalent (*E.C.*) chambers by the gills (*G.*), A in *Nucula*, B in a typical Eulamellibranch. Direction of water current indicated by arrows passing through gills, direction of food streams by arrows on surface of gills, forwardly directed streams to mouth indicated by *X*. In B the ascending and descending filaments of each lamella are drawn apart for the sake of clearness. (Original.)

been the subject of a fine paper by Ridewood (1903) and their function most thoroughly studied by Wallengren (1905), Orton (1912, 1913, 1914), Kellogg (1900, 1915), Allen (1914), Yonge (1923, 1926 a), Churchill and Lewis (1924) and Nelson (1924). They divide the mantle cavity into a ventral, sometimes anterior, inhalent chamber (*I.C.*) and a dorsal, sometimes posterior, exhalent chamber (*E.C.*), as shown diagrammatically in Fig. 9, between which they interpose a sieve of tissue (*G.*) through which water is strained leaving suspended particles on the surface. The gills of the Protobranchs, such as *Nucula* (A), consist of single series of flat oval leaflets

one on either side of the body attached side by side along an axis on the middle of the dorsal surface, bundles of large cilia at either extremity effecting junctions with the mantle at the side and the other series of leaflets in the middle (Fig. 11, *o.c.d.*, *l.c.d.*). In the Filibranchs and Eulamellibranchs, the gills consist of lamellae formed of many fine filaments, oval in cross-section, each lamella being formed of
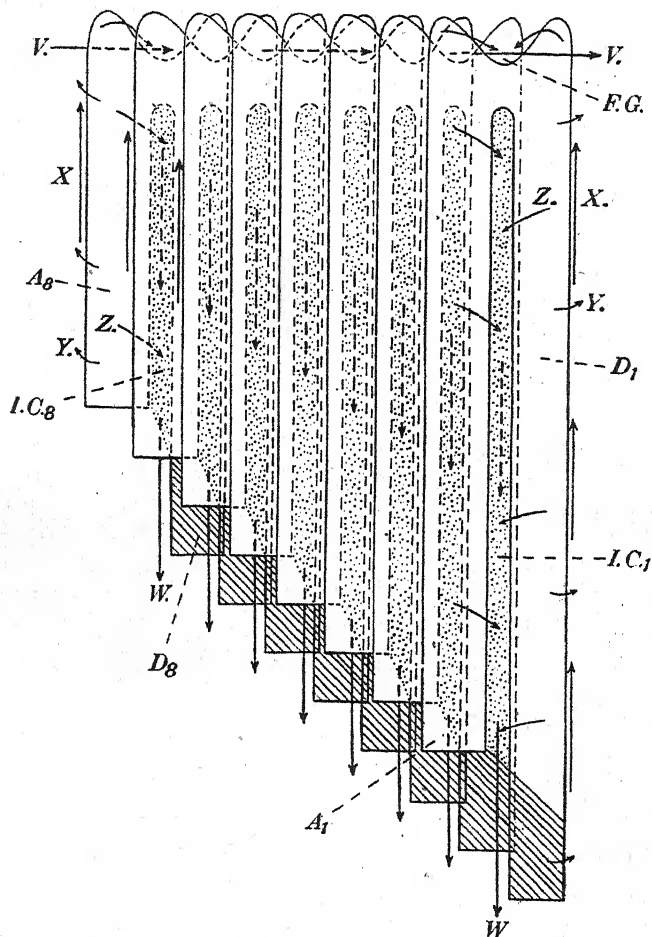
Fig. 10. Diagram showing structure and ciliary currents of Filibranch or Eulamellibranch gill lamella (ciliated discs or interfilamentary junctions, and interlamellar junctions not shown). A_1, A_8, first and last ascending filaments; D_1, D_8, first and last descending filaments; F.G. food groove; I.C. 1, I.C. 8, interlamellar cavities between ascending and descending filaments; V. direction of current in food groove, leads to mouth; W. direction of current in interlamellar cavity; X. direction of beat of frontal cilia; Y. direction of latero-frontal cilia; Z. direction of lateral cilia. (Original.)

ascending and descending filaments uniting at the free extremity, as shown in Fig. 10. In the Protobranchs and Filibranchs, the leaflets or filaments are united laterally by "ciliated discs" (Fig. 11, *c.d.*) but in the Eulamellibranchs there are interfilamentary junctions uniting adjacent filaments. In the Filibranchs and

Eulamellibranchs the two halves of each lamella are united by interlamellar junctions. In all but the Protobranchs the gills of either side usually consist of two lamellae or demibranchs, as shown in Fig. 9; details of the exceptions to this are provided by Ridewood.

The water current is produced by the action of lateral cilia on the gill filaments. Side views of single filaments of the gills of *Nucula* and *Mytilus* (Filibranch) are shown in Fig. 11, the distribution and direction of beat of the cilia being indicated. The lateral cilia (*l.c.*) lash across the surface of the filaments and so cause a current of water (*Z.*) to flow through the narrow slits between the filaments, from the
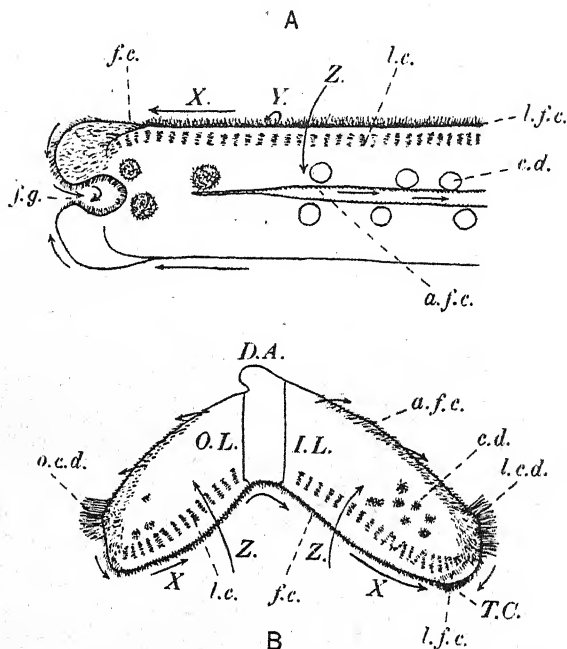
Fig. 11. Lateral and anterior views respectively of A, living filament of outer lamella of gill of *Mytilus*, and B, living pair of leaflets of right gill of *Nucula*. *a.f.c.* abfrontal cilia; *c.d.* ciliated discs; *D.A.* dorsal surface of gill of *Nucula*, where attached; *f.c.* frontal cilia; *f.g.* food groove; *l.c.d.* cilia affecting junction with similar cilia on left gill; *I.L.* inner leaflet of gill; *l.c.* lateral cilia; *l.f.c.* latero-frontal cilia; *o.c.d.* cilia affecting junction with mantle; *O.L.* outer leaflet; *T.C.* cilia which carry food forwards; *X.* direction of beat of frontal cilia; *Y.* direction of latero-frontal cilia; *Z.* direction of lateral cilia. (From Orton (1912).)

inhalent, into the exhalent chamber, directly in the case of the Protobranchs but by way of the interlamellar cavity (between the ascending and descending filaments, see *I.C.* Fig. 10), in the other Lamellibranchs (*W.*). In the Protobranchs and Filibranchs the formation of this current is aided by the beating of abfrontal cilia (*a.f.c.*) on the inner surface of the filaments, but in the Eulamellibranchs, where the lamellae are much firmer, these cilia are unnecessary and so absent.

The lateral cilia never vary in position or direction of beat in the different species, but the food collecting cilia are not so standardised. The frontal cilia (*f.c.*) which cover the outer, exposed surface of the filaments are the chief agents

of food collection. In the Protobranchs, *Nucula* (Orton, 1912) and *Yoldia* (Kellogg, 1915) the frontal cilia carry particles to the middle line whence they are conducted in two parallel streams towards the mouth (Fig. 9A, *X*.). In *Solenomya* (Orton, 1913) they also lash ventrally but, owing to the different arrangement of the gills, the two forwardly directed tracts are widely separated, one at either side of the mantle cavity. In the other Lamellibranchs the frontal cilia usually beat towards the free, lower margin of the lamellae (see Figs. 10 and 11 (*X*.)) where there is a groove (Figs. 10, *F.G.* and 11, *f.g.*) in which particles are carried anteriorly (Fig. 9B, *X*.). In *Pecten* (Kellogg, 1915) and *Ostrea* (Yonge, 1926 *a*) the frontal cilia on the large principal filaments in the grooves between the ridges or plicae of filaments beat in the opposite direction. In this manner a certain selection of particles is made, only the smallest particles falling into the grooves and being carried to the base. There are forwardly directed ciliated tracts on both sides of the base of the lamellae (Fig. 9B, *X*.), the epithelium being especially modified in these regions (see Yonge, 1923, Fig. 7). In *Anodonta* (Wallengren) all the frontal cilia on the outer demibranchs of either side beat towards the base. All these types are represented diagrammatically in Fig. 9B. The frontal region of the filaments is plentifully supplied with mucus glands in the secretion of which the food particles are entangled. Among the small frontal cilia are occasional large "cirri" (Wallengren) which are also found in all regions concerned with the transport of food. At the edge of the filaments, between the frontal and lateral cilia, are large laterofrontal cilia (Fig. 11, *l.f.c.*) which lash cross ways (*Y*.) on to the frontal region. The latero-frontals of adjoining filaments interlock and these cilia act largely as strainers, preventing particles passing between the filaments in the water current and throwing them on to the frontal cilia. As a result of the action of the various sets of cilia on the gills a continuous current of water is created, and all suspended particles are intercepted and conveyed either towards the mouth or out of the mantle chamber.

There is also a certain selective action on the gills, in *Pecten* and *Ostrea* by the difference in the beating of the frontal cilia on the principal filament from those on the other filaments, and in *Monia* (Kellogg, 1915) by a division of the frontal cilia on all filaments into a narrow strip near the base which beat in that direction, matter being subsequently conveyed to the mouth, and those on the remaining surface which all beat towards the free margin, the "food groove" of which contains cilia beating *posteriorly*, all matter which comes under their influence being eventually rejected. Moreover, large particles or masses in the groove at the margin of the lamellae are liable to fall over on to the surface of the mantle whence they are removed. It has further been noted by Kellogg (1915) in *Pecten* and Yonge (1926 *a*) in *Ostrea* that *muscular* contractions of the gills cause material to be transferred from the grooves on to the crests of the plicae and from the surface of the gills to that of the mantle. This muscular action is probably more widespread and more important than has hitherto been recognised.

Palps.

The gills are relatively small in the Protobranchs, being concerned primarily with the creation of the water current; in these animals the palps are directly concerned with food collection whereas in the other Lamellibranchs they are selective mechanisms sorting out the material passed on to them from the gills. In the Protobranchs, the palps are relatively large and vary in structure in the different genera. They consist of three parts which, adopting the terminology proposed by Hirasaka (1927), may be called the palp proboscis (Fig. 12, *Pb.*), an extensile organ present in all Protobranchs and their chief organ of food collection, the palp lamella (*Lm.*) which corresponds to the labial palps of the other Lamellibranchs but is absent in *Solenomya* (Pelseneer, 1891, Morse, 1913), while in *Nucula* there is also a palp pouch (*Po.*) between the proboscis and lamella.

In life the palp proboscis may be extended for a considerable distance outside the shell and is very sensitive and active especially near the tip. Its under surface
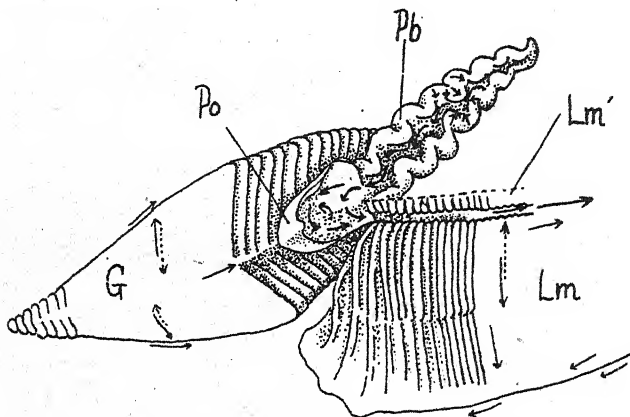
Fig. 12. Diagrammatic sketch of palp feeding mechanism of *Nucula nucleus*, ventral view, showing direction of ciliary current on right side; outer right lamella almost removed (*Lm′.*). Broken arrows show ciliary currents in deeper zones. *G.* gill; *Lm.* palp lamella; *Pb.* palp proboscis; *Po.* palp pouch. (After Hirasaka (1927), Fig. 4. By kind permission of the Marine Biological Association.)

is grooved and ciliated and, as the proboscis moves about on the surface of the sand or gravel, particles are caught in the ciliary current which carries them along the groove towards the base. This food collecting function of the proboscis was surmised by Mitsukuri (1881) and established for *Yoldia* by Drew (1899 *a*) and Kellogg (1915), for *Nucula* by Drew (1899 *b*), Morse (1919) and Hirasaka, and for *Solenomya* by Morse (1913). Particles are passed to the palp lamella, directly in *Yoldia* (see Kellogg, 1915, Fig. 70) and by way of the palp pouch in *Nucula* (Hirasaka) which changes their direction depositing them at the posterior end of the lamella, as shown in Fig. 12. The lamellae are ridged transversely; according to Hirasaka there are three zones of cilia on the ridges in *Nucula*, those on the summits beating ventralwards and carrying particles to the lower edge of the palp whence they are rejected, those in the middle being especially large and having, Hirasaka thinks,

selective powers, while those in the furrows beat dorsalwards and carry food to the dorsal margin of the palp where it is caught in currents which lead towards the mouth. Kellogg (1915) has described the dorsal beating of the cilia on the ridges of *Yoldia* but does not mention any ventrally beating cilia, stating that the remaining cilia beat anteriorly carrying particles transversely across the ridges towards the mouth. Food is conveyed to the palp lamellae from the gills in small quantities in *Nucula* (Hirasaka) and in larger amounts in *Yoldia* (Kellogg) where there is a special extension of the "lateral oral groove." In Protobranchs generally, however, the palp proboscis and *not* the gill is the chief organ of food collection.
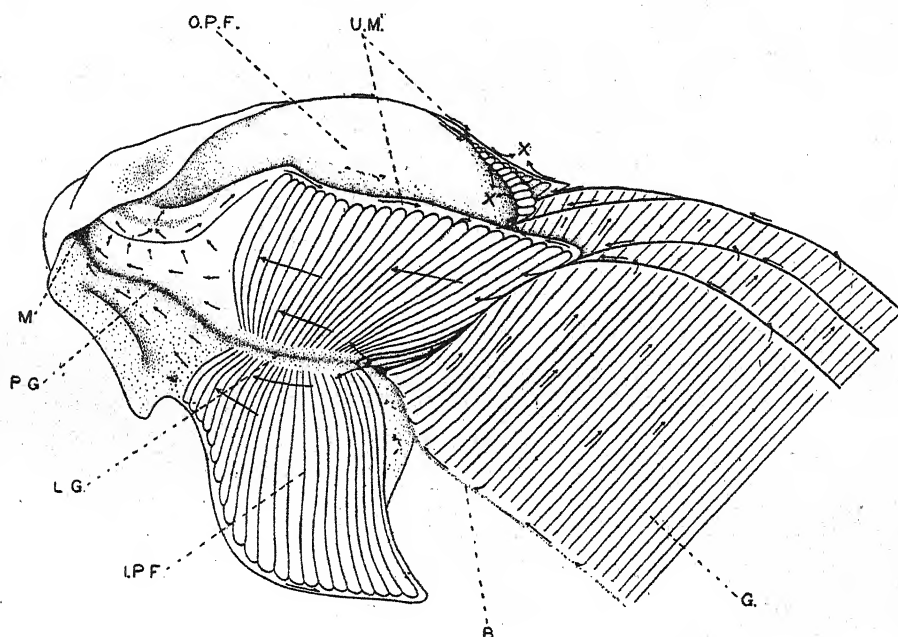
Fig. 13. *Ostrea edulis*, junction of palps and gills, right palps opened out so as to expose inner ridged surfaces. ×8. *B.* base of gill demibranch; *G.* gill; *I.P.F.* inner palp face; *L.G.* lateral oral groove; *M.* mouth; *O.P.F.* outer palp face; *P.G.* proximal oral groove; *U.M.* upper margin of palps; *X.* point where material is rejected from palps. (After Yonge (1926 *a*), Fig. 1. By kind permission of the Marine Biological Association.)

In the Filibranchs and Eulamellibranchs the palps are arranged, as in the Protobranchs, in two pairs, one on either side of the mouth; but they have no appendages. As shown in Fig. 13, their outer surfaces (*O.P.F.*) are smooth, and their inner opposed surfaces (*I.P.F.*) ridged transversely. They vary greatly in size and shape in different genera (see Kellogg, 1915, for descriptions and figures of many of these) but generally speaking are triangular flaps. The gill lamellae arise either just between the posterior ends of the opposed palps or immediately posterior to the palps, material being passed on to the surface of the palps from the ciliary currents on the summits and along the axes of the demibranchs, as shown by the arrows. The groove between the folded region of the palps may be termed the

lateral oral groove (*L.G.*), that leading from it to the mouth (*M.*), anterior to the folded region, the proximal oral groove (*P.G.*) while, in animals where the outer demibranch does not extend so far forward as the inner, there is a distal oral groove posterior to the lateral one (Kellogg).

The palps are highly developed sorting organs and their function has been especially studied by Wallengren (1905), who was the first to give an accurate account of their diverse ciliary currents, Kellogg (1915), Allen (1914), Yonge (1923, 1926 *a*), Nelson (1924) and Churchill and Lewis (1924). The outer, smooth surfaces (*O.P.F.*) are covered with small cilia which carry particles diagonally backwards, their function being that of cleansing. There is a powerful backwardly directed
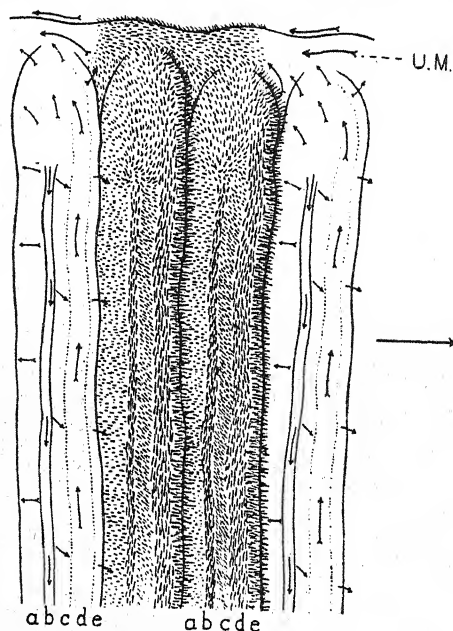
Fig. 14. Semidiagrammatic figure of palp folds of *Ostrea*, showing direction of ciliary currents. × 60. *a, b, c, d, e*, tracts of cilia on exposed surface of folds; *U.M.* upper margin. Large arrow shows direction of mouth. (After Yonge (1926 *a*), Fig. 25. By kind permission of the Marine Biological Association.)

current (*U.M.*) along the upper margin of the inner surface of the palps in *Ostrea* and along the anterior edge in the majority of Lamellibranchs where the body has its greatest axis anterio-posteriorly (the two being morphologically identical). In it particles are carried to a point at, or near, the tip (*X.*) where they are caught in a vortex, rolled round and finally rejected by way of the mantle. The ciliation of the ridges, which do not extend quite to this margin, is very complicated and will be most easily described by reference to Figs. 14 and 15.

Each fold bends forward towards the mouth, overlapping the base of the fold immediately anterior to it. Down the centre of the exposed distal surface of each fold runs a longitudinal groove. There are five ciliated tracts on the *exposed* surface of the folds in *Ostrea* (Fig. 14), most distally there is a tract (*a*) the cilia of which

beat downward into the furrow between adjacent folds, but this region is largely overlapped by the more distal fold; next there is a narrow tract (*b*) within the longitudinal groove whose cilia beat towards the lower surface of the palp (posterior in the majority of Lamellibranchs); then a narrow tract (*c*) which directs particles diagonally across the palp towards the mouth; then a tract (*d*) in which particles are carried to the upper (in other cases anterior) margin of the palp; and finally a tract (*e*) whose cilia beat in a direction at right angles to the line of the folds and towards the mouth. As shown better in Fig. 15, in which two folds of the palps of *Mya* are shown drawn apart with the furrow (*F*.) and the proximal surface (*p.s.*) of the more posterior fold exposed, there are important tracts of cilia in the furrows in which particles are conveyed to the posteriorly directed marginal tracts, while
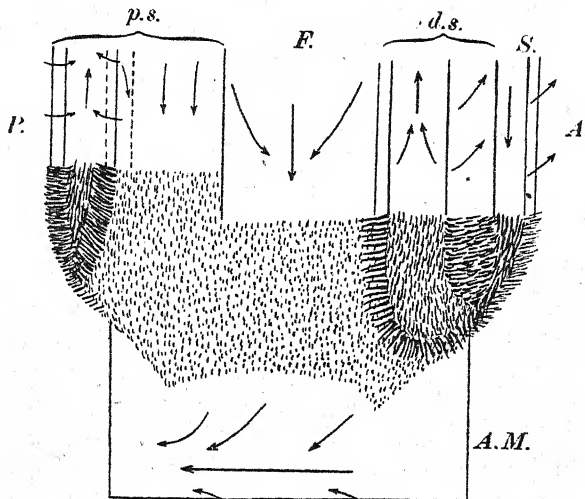
Fig. 15. *Mya arenaria*, portion of inner surface of palps showing anterior margin (=upper margin in *Ostrea*) and beginning of two folds, posterior one turned back to display the proximal surface. *A.* anterior; *A.M.* anterior margin; *d.s.* distal slope of anterior fold; *F.* furrow between folds; *P.* posterior; *p.s.* proximal slope of posterior fold; *S.* summit of anterior fold. Arrows indicate direction of beat of cilia, relative sizes of which are shown. (From Wallengren (1905) and Yonge (1923).)

on the proximal surface there is a deep tract leading in the same direction as the furrow tract, and a narrow band of cilia beating towards the summit of the fold and throwing particles into a tract of cilia whose beat is in the opposite direction to that of the cilia in the furrows. Details of the palp ciliation vary, however, in different species (see Wallengren).

The path of particles across the palp is largely the resultant of the action upon them of these different tracts of cilia, the interaction of which is very difficult to investigate; but there can be no doubt that the whole forms an extraordinarily efficient sorting mechanism. When particles are placed upon the inner surface of the palps of *Ostrea*, the effect of the five exposed ciliated tracts is that light particles such as carmine grains are thrown lightly from fold to fold by the action of the especially large cilia of tract (*e*), but large particles such as carborundum, or small

particles massed together in big strings of mucus, tend to be drawn down within the furrows under the action of the cilia in tract (a) and thence expelled. The mucus is of great importance for the more there is secreted, either round a large number of fine particles or one or more larger particles, the greater the chance of these being drawn into the furrows and rejected.

Muscular action is also of the greatest importance. Both Wallengren and Kellogg report the muscular retraction of the proximal edges of the folds, particles being thereby allowed to fall into the furrows; in *Ostrea* this is not so obvious, but here, as in other Lamellibranchs, there is an immediate reaction when large particles are placed on the palp surface, the entire palp curling back in the manner shown in Fig. 13. As a result the folds are drawn apart and the furrows with their outgoing tracts of cilia exposed. The palps occasionally curl inwards so that the folds are puckered and material falls into the furrows through the spaces so provided. In *Schizotherus*, Kellogg states that the ventral (anterior) margin of the palp curls over and draws off material from the palp surface. Cobb (1918) found that the palps of *Anodonta* respond by muscular contractions to mechanical, electrical, chemical, photic, and thermal stimuli. Churchill and Lewis (1924) and Nelson (1924) have observed muscular movements in the palps of young mussels and oyster spat respectively. Yonge (1926 a) found that in the spat of *Ostrea edulis* the palps were relatively much larger than in the adult, being very active and responding to stimuli by drawing back and upwards thereby exposing outgoing tracts.

The great majority of recent workers are agreed that feeding in Lamellibranchs is purely *quantitative*, the particles taken into the mantle cavity being subjected to the series of selective mechanisms already referred to as a result of which large particles or mucus laden masses are rejected and smaller particles or masses are passed to the mouth, quite irrespective of their food value.

Ciliary currents in the mantle cavity.

In *Ostrea*, as shown in Fig. 8, material dropped on to the mantle surface is caught in ciliary currents which remove it from the body. There are tracts in the anterior region which conduct particles to a point about the middle of the inhalent aperture where they accumulate under the thickened, unciliated ridge which bounds the mantle; from time to time these masses are expelled by sudden contractions of the valves. In the posterior region of the inhalent chamber and in the exhalent chamber matter passes direct to the edge of the mantle. In siphonate Lamellibranchs such as *Mya* (Yonge, 1923), where the mantle folds are united for the greater part of their extent, material is carried back in a ciliated tract situated in the mid-ventral line as originally described by Stenta (1901, 1903). Where siphons are present, the waste material accumulates at the base of the inhalent siphon through which it is periodically expelled by sudden contractions of the adductor muscles. There are many variations of these rejective mechanisms, details of which are given by Kellogg (1915). It is notable that in young, spat oysters, where the danger of silting up is extremely great, the rejective mechanisms are more highly developed than in the adult (Yonge, 1926 a).

For the sake of completeness, a short account of ciliary feeding mechanisms in the primitive chordate groups is given below.

Hemichordata. Although no observations appear to have been made on living *Balanoglossus*, Orton (1913) found well-marked lateral and smaller frontal or pharyngeal cilia in sections of the gill-bars from which he deduced, with apparent reason, that the former produced the main current through the body and the latter were concerned with the collection of food. According to Gilchrist (1915), all parts of the body and stalk, both of zooids and buds, of *Cephalodiscus gilchristi* are covered with cilia by means of which particles are carried to the arms. These possess larger cilia which beat distally except those in the central, broad and shallow grooves which are especially long and beat towards the mouth. There appears to be some selection of particles, in which the tentacles—feebly ciliated but freely movable—take part.

Tunicata. The feeding of Ascidians has been described in most accurate detail by Fol (1876), Roule (1884) and Orton (1913) all of whom are in close agreement. By the action of the cilia on the sides of the gill-bars of the branchial sac, an inhalent current is produced through the branchial opening, particles in suspension are caught on the surface of the sac while the water passes through the fine mesh-work into the atrium, leaving the body as an exhalent current through the atrial aperture. Food is collected by the cilia on the pharyngeal surface of the gill-bars and on their papillae which carry it across the surface from the endostyle towards the dorsal lamina. In the simple Ascidians this process is assisted by a transverse waving of the longitudinal bars. The cilia lining the endostyle beat outwards and so transfer the mucus there secreted on to the surface of the pharynx. The mucus-laden food masses eventually reach the dorsal lamina, in which they are carried towards the posterior end of the branchial cavity, whence they are transported *forwards* into the oesophageal opening.

Fedele (1923) considers the process of feeding in *Doliolum* to be the result of the co-ordination of secretory, ciliary, muscular and nervous activities. The endostyle secretes the entangling mucus which is carried dorsally by the peripharyngeal cilia and directly to the oesophagus by cilia on tracts between it and the endostyle. The cilia on the gill-bars produce vortices which create an ingoing current, while those in the alimentary canal, besides passing food through the gut, draw in mucus-laden masses from the branchial sac. The circular muscle bands by contracting regulate the current produced by the cilia, assist in the trituration of particles within the pharynx and, by more sudden movements, assist ingestion in the pharynx and ejection in the atrium. There is a nervous control of ciliary and muscular movements, both of which are intermittent.

A very remarkable state of affairs is found in the Appendicularians which collect food by means of a gelatinous "house" which they secrete about themselves, and only secondarily by ciliary action. One of the most elaborate of these is formed by *Oikopleura*, the structure and function of which have been described by Lohmann (1899). By the lashing of the tail, water is drawn into the "house" on the dorsal side through a pair of funnels whose external openings are provided with a fine

meshed grating which only allows the finest particles or nannoplankton—with a maximum diameter of 1/30 mm.—to enter. The cavity within the "house" is divided into dorsal and ventral chambers, the funnels open into the latter and water is then drawn through an elaborate collecting apparatus consisting of paired wings and a median, unpaired portion. The cavity of these wings is divided by the membrane separating ventral and dorsal chambers; water enters the ventral division on either side and the two streams meet in the middle line when the water passes dorsally through the dorsal divisions of the wings in which are many fine septa, which effectively sieve out all suspended matter which remains in the median channel whence it is sucked into the pharynx of the occupant of the "house" by ciliary action. The sieved water passes into the ventral chamber eventually reaching the posterior end of the "house" where there is a small opening, normally closed by muscles, which is forced open when the pressure of water rises above a certain point. By the ejection of water the animal is forced forward, but the main function of the "house," as Lohmann has pointed out, is food collection not locomotion. Unlike the other Tunicates, therefore, ciliary action in the Appendicularians is confined to the drawing in of food previously collected by a specially constructed sieving apparatus, the necessary current being created by the muscular movements of the tail. The "house" quickly becomes clogged with particles and so useless, it is then abandoned by the animal—after perhaps only a few hours—and a new one constructed in from a quarter to half an hour.

Amphioxus. Orton (1913) has described the feeding of *Amphioxus*. A stream of water, from which the largest particles are removed by the buccal tentacles, is maintained through the pharynx by the beating of two rows of lateral cilia on the gill-bars, particles being entangled in mucus secreted by the endostyle and carried over the surface of the pharynx by the cilia on the pharyngeal surface of the gill-bars. The same cilia conduct the food, which they work up into cylindrical masses with mucus, into the dorsal groove, the cilia of which transport it posteriorly into the alimentary canal. The water passes into the atrium and thence to the exterior through the atrial opening. There is also a minor collection of food in the buccal cavity by the ciliated "wheel organ" and Hatchek's pit, particles being caught in mucus and carried to the dorsal groove by way of the peripharyngeal bands, as previously noted by Andrews (1893).

Ciliary feeding by larvae.

The free-swimming larvae of many animals feed by means of cilia even though the adults do not. Thus *Asterias* and similar Echinoderm larvae collect food on the longitudinal ciliated bands whence it is carried to the dorsal border of the stomadaeum, excess matter being expelled from the ventral end, in the opinion of MacBride (1914, p. 463), by the cilia on the adoral band. The pilidium of the Nemertines, the trochophore of the Annelids and the veliger larvae of the Molluscs, all feed by ciliary currents. The feeding of the larva of *Ostrea edulis* has been described by Yonge (1926 a). The large cilia of the velum throw particles on to a ciliated tract round the base in which they are carried to the mouth, excess

matter being removed by outgoing tracts of cilia on the rudiments of the foot. It is possible that the ammocoete of *Petromyzon fluviatilis* may feed in a similar manner to *Amphioxus*; Orton (1913) fed specimens with carmine, and later found particles entrapped in mucus in the branchial region and collected along the gill-bars and the roof of the pharynx, but he did not find carmine in the alimentary canal.

(c) Tentacular.

A few animals collect finely divided food with the aid of freely movable tentacles.

Echinodermata. The Dendrochirote Holothuroidea, such as *Cucumaria*, *Thyone* and *Psolus*, have a crown of branching tentacles round the mouth. Their method of feeding has been described by many authors (see Ludwig, 1889 for earlier references), most recently by Pearse (1908), whose account of the feeding of *Thyone* is very detailed, and Hunt (p. 570). They live in cracks in rocks or bury themselves in mud and entangle plankton and other fine particles on the tentacles which are widely extended and covered with adhesive slime. The tentacles are then thrust at regular intervals, one after the other into the mouth and then immediately withdrawn, the adherent matter being wiped off against one of the two small, bifurcate, ventral tentacles.

Annelida. Certain tubicolous and burrowing worms, members of the Terebellidae, Amphictenidae, Ampharetidae, Chlorhaemidae and Spionidae (see Blegvad and Hunt), are most conveniently considered here. They possess extensile cephalic tentacles which grope about on the surface of the bottom deposits. Each is furnished with a longitudinal, ciliated furrow on the under side, and by means of continuous movements of the tentacles, particles and small organisms are caught in this groove and carried to the mouth. Although cilia are employed, the groping movements of the tentacles are of equal, or greater, importance in feeding.

Mollusca. The filamentous capitacula around the mouth of *Dentalium* are probably concerned with the collection of fine food particles.

(d) Mucoid.

The solitary example of this type is provided by the interesting Gastropod, *Vermetus*, whose feeding habits have been described by Simroth (1901 a). The animal possesses a very large pedal gland, useless for locomotion as the animal is sessile, which secretes a veil of mucus extending outwards from the mouth into which it is from time to time withdrawn together with entangled food particles.

(e) Muscular.

Unlike many Scyphozoa which capture large prey, *Rhizostoma* takes in fine particles which, according to von Uexküll (1901), it sucks in by muscular action. The four arms of the other Scyphozoa are here subdivided to form eight, all of them foliaceous and possessing at their extremities many fine perforations which lead into the longitudinal canals opening into the stomach. As a result of the pulsations of the bell, the stomach is alternately increased, when water and finely divided matter are drawn in, and decreased in size, water only being expelled.

(f) Setous.

The collection of minute food particles by means of chitinous limbs fringed with fine setae is widespread amongst the aquatic Arthropods[1].

Crustacea. Here, as in the Mollusca, fine particle feeding has been developed in many groups, apparently independently of one another.

Branchiopoda. In *Simocephalus vetulus*, Cannon (1922) states that a food-stream is drawn in by the outward movements of the endites of the third and fourth trunk limbs which, since they are not placed vertically but are slightly further apart at their distal than at their proximal end, probably "causes a small backwash in a forward direction in the food groove." At the tip of the labrum suspended particles are removed by the setae on the gnathobases of the second trunk limbs and brushed dorsally into the channel formed by the tip of the labrum and the maxillae. The anterior three setae on the gnathobase are comb-like and brush on to the food lying between the maxillae the secretion of the labral glands, which open near the tip of the labrum. The secretion contains no mucus but is sticky and probably binds together the particles which are then pushed between the mandibles by the anteriorly directed setae on the maxillae. In Daphnids, according to the account given by Storch (1924, 1925 a, 1925 b) and represented schematically in Fig. 16, a stream of water (1) is drawn in from the antero-ventral region by the action of a pump formed by the body wall (dorsally), the shell and legs (laterally) and ventrally by the more distal regions of the third and fourth trunk limbs (III, IV) which are placed almost vertically with their setae pointing towards the mid-ventral line. By the movements of these legs the chamber is enlarged and water sucked in (4), it is then narrowed, the distal edges of the limbs coming together before the more proximal, fringed regions, and in this way, accord-ing to Storch, water is forced either into the food groove (5) which runs along the mid-ventral line or else laterally into outlet chambers lying between these appendages and the shell, fine particles being filtered off by the fringing setae in the process.

Fig. 16. Schematic representation of the feeding of *Daphnia*. 1, inhalent stream; 2, exhalent stream; 3, vortex between 1 and 2; 4, vertical stream between limbs; 5, forward streaming of food in food groove; 6, accumulation of food in front of mouth; 7, mandible; III, IV, edges of the third and fourth trunk limbs. (From Storch (1924).)

The pump movements take place four or five times per second. Filtered water is rejected from the postero-ventral region (2), while the food particles (6) are carried

[1] Among the Annelida, Cori (1923) has shown that the tube-dwelling, freshwater oligochaete *Ripistes* collects fine particles on the especially long setae of segments 6–8, whence they are removed by backward movements of the head, the ventral setae on the second segment acting as a comb.

to the mouth by means of the anteriorly directed setae on the gnathobases of the second trunk limbs. There is apparently no mastication of particles. A very similar account is given by Franke (1925) for *Chydorus*.

Cannon and Manton (1927) criticise Storch's views, being unable to find evidence that particles are deposited on the inner surface of the setae on the trunk limbs which they consider merely prevent particles from escaping from the central filter chamber. They also disagree with him as to the function of the gnathobases of the second trunk limbs, upholding Cannon's earlier view that particles are carried forward in the food groove as a result of the backwash caused by the outward, and to some extent the inward, movement of the limbs. The setae on the gnathobases of the second trunk limbs concentrate the particles and sweep on to them the secretion of the labral glands. Storch is uncertain of the function of these glands but Cannon appears to have excellent reason for considering them of vital importance in feeding, for without the binding action of the secretion the risk of isolated particles being swept away would be great. In the nauplius of *Estheria*, Cannon (1924) states that the labial glands are precociously developed; the food current is drawn into the mouth beneath the labrum and, as there are only the single masticatory setae on the antennae for pushing in the food, there is an obvious need of some substance to bind together the particles.

Naumann (1921) describes the feeding currents of a number of Cladocera, especially *Sida*, *Daphnia* and *Bosmina*, but in less detail; he recognises the main, backwardly directed current but considers the food is filtered through the setae on the trunk limbs, the food being then carried forward in the food groove towards the mouth.

Ostracoda. The feeding mechanisms of Ostracods, as typified by *Pionocypris vidua* and *Notodromas monacha*, have been studied by Cannon (1926) and Storch (1926) respectively. Cannon's account is the more detailed and will be followed here. Food in the form of bottom deposits is kicked up by the antennae and a stream of water with suspended matter is maintained antero-posteriorly through the shell by two vibratory plates, a small one in the anterior chamber and a larger and more important one in the posterior chamber of the shell, the former being part of the mandibular palp and the latter the epipodite of the maxillule. The plates oscillate at the same speed but never move in the same direction at the same time; the anterior part of the maxillulary plate remains almost stationary but the posterior part moves up and down, the action resembling that of "an oar used in sculling over the stern of a boat." In many Ostracods there is a third important vibratory plate on the posterior part of the maxilla.

The food current enters between the antennules and the mouth and is immediately divided into two by the labrum; these streams are concentrated as they enter the narrow passage between the adductor muscle and the edge of the shell which leads into the posterior chamber, and the suspended matter is here collected by the terminal hairs on the mandibular palps which swing continuously backwards and forwards. The food is then gripped by the terminal setae of the maxillules which work in conjunction with the palps. Food collected directly by the maxillae

and the antennae is also passed on to the maxillules. Labial glands are present and Cannon suggests that they produce a viscid secretion in which the food on the maxillules is entangled. It is certainly difficult to see how else they could escape being washed away. Food is transferred dorsally from the maxillules to the mandibles by a pair of complicated "food-rakes"; the mandibles act irregularly, food being probably passed into the oesophagus with the help of recurved spines on the dorsal region.

Copepoda. Esterly (1916) was unable to determine exactly how the food currents are produced in *Calanus* but saw that they come either from the front of the animal and pass between the bases of the anterior antennae, or from behind the bases of the posterior maxillipeds, from which particles are directed towards the mouth by the long spinose bristles on the anterior maxillipeds. These form a funnel, the food being formed into a pellet at the narrow, anterior end which is overhung by the upper lip and thence pushed into the mouth by the bristles on the inner lobes of the maxillae.

In *Diaptomus* (see Fig. 17), according to Storch and Pfisterer (1925), the food stream (1), which begins some distance anterior to the body, is created by the backward movements of the second antennae (*A.*2), the endites of the mandibles (*Md.*) and the first maxillae (*Mx.*1); it reaches the trunk region (2) after passing through the outer branches of the first maxillae and here draws water (3) from between the first and second maxillae (*Mx.*2) which, in its turn, produces a current from behind into the "filter-chamber" between the second maxillae and the maxillipeds (*Mp.*) of each side (4). Food particles are filtered by the bristles of the second maxillae and collected at the narrow end of the filter chamber by the combing action of the endites on the first maxillae which carry them to the mouth. Thus the Copepods appear to possess a true filtering apparatus which, according to Naumann (1923), is so efficient in *Diaptomus* that particles less than 1μ are retained.
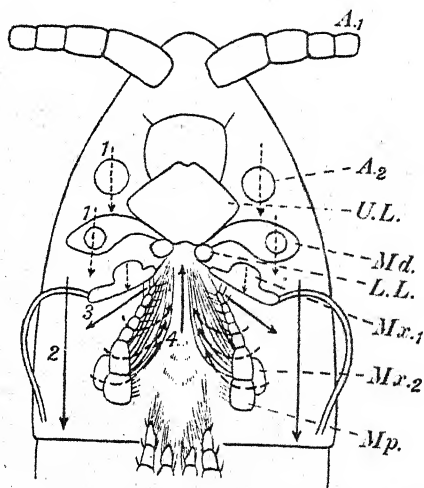
Fig. 17. *Diaptomus*, diagram showing food currents and mouth parts. A.1, first antenna; A.2, second antenna; *L.L.* lower lip; *Md.* mandible; *Mp.* maxilliped; *Mx.*1, first maxilla; *Mx.*2, second maxilla; *U.L.* upper lip; 1–4, food currents, explained in text. (From Storch and Pfisterer (1925).)

Cirripeda. The Cirripedes feed largely on minute Crustacea which they collect with the six pairs of thoracic legs or "cirri," each consisting of a basal piece and two long rami bearing many long hairs. These sweep like a casting net through the water entrapping all small animals and suspended particles within range. Gruvel (1893) has perhaps described the process most carefully. Food is deposited by the cirri between the maxillae and later passed to the mandibles, the

movements of which can be simultaneous or alternate according to requirements. After mastication the food is worked into round masses which are swallowed.

Mysidacea. Depdolla (1923), working on *Praunus flexuosus*, found that not only could this Mysid deal with large food particles, but that it could also collect suspended matter. He states that two symmetrical water streams approach the antennal scale anteriorly, inclined at an angle of 30° to the longitudinal axis, and unite to flow posteriorly between the thoracic endopodites, fine particles carried near the mouth being caught by the first and second trunk limbs and by the maxillae. Cannon and Manton (1927), in their detailed and beautiful account of the feeding of *Hemimysis Lamornae*—a smaller but structurally very similar animal—though they agree with Depdolla as to the method of feeding on large particles, give a very
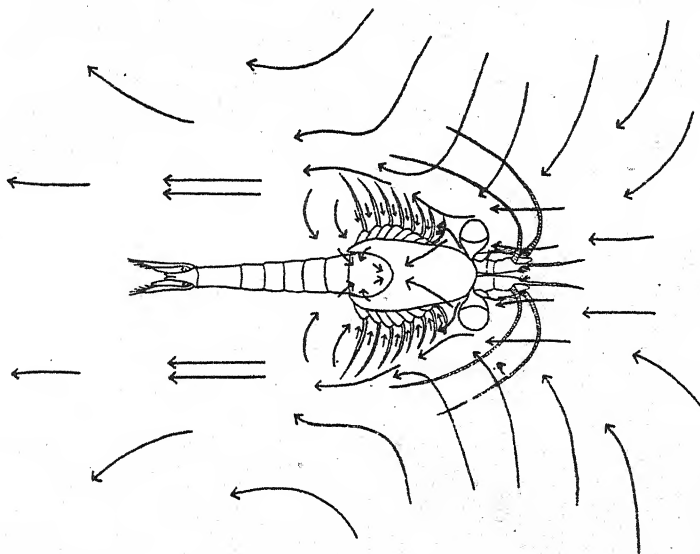
Fig. 18. *Hemimysis Lamornae*, dorsal view of the animal swimming freely showing the currents produced in the surrounding water. ×6. (After Cannon and Manton (1927), Text-fig. 5. By kind permission of the Royal Society of Edinburgh.)

different description of the filter-feeding. Water currents responsible for locomotion and feeding are produced by the thoracic exopodites which are whirled rapidly round so that their tips describe a series of ellipses (Fig. 19A). Their lateral setae spread out on the upward and backward stroke but collapse as they pass forward, the animal being driven onward by the former movement. The resultant currents are shown in Fig. 18. A food stream is produced by each thoracic exopodite (see Fig. 19B); "The rotating whip-like limb causes a conical swirl with the apex at the base of the limb, which draws water towards it from all directions" (p. 228), for in whatever position the exopodite is it tends to push water before it and so causes a backwash round the sides and upward into the area of lower pressure in the proximal area of the limb. After passing between the limb bases, the streams join to form a forwardly directed current in the channel formed by the inner basipodites

4-2

and the ventral body wall. This current is probably produced by three agencies: (1) the pressure of the water current created by the exopodites, the shape of the limb bases giving it a forward motion, (2) the exhalent respiratory current which passes out at the sides of the maxilla and maxillule, and (3) the movement of the whole maxilla and its exite.

On reaching the widened food-basin near the maxilla the stream divides into two, which pass out laterally behind the bases of the paragnaths and through the overlapping combs of setae of the basal endites of the first trunk limbs and the
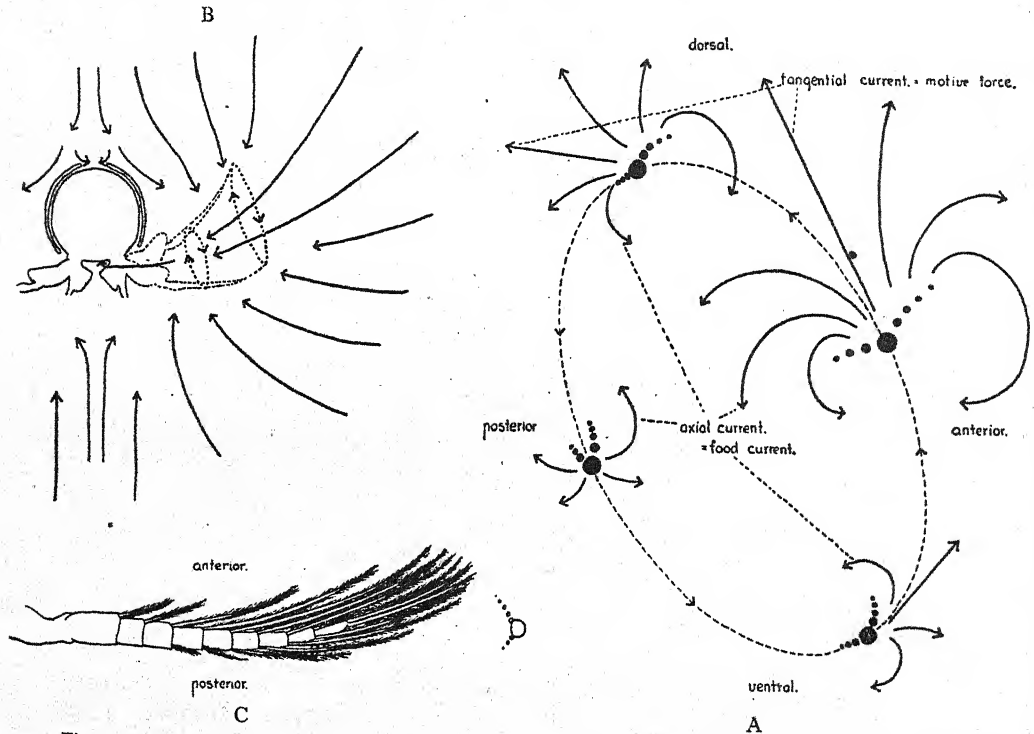
Fig. 19. *Hemimysis*. A, diagram representing tip of single exopodite as it is whirled round, showing positions of setae and axis and created water currents at various phases of the beat. B, diagram indicating thoracic transverse section of animal, showing rotation of one exopodite with water currents produced. C, dorsal view of single exopodite, showing arrangement of lateral setae, also diagrammatic section across limb. (After Cannon and Manton (1927), Text-fig. 6. By kind permission of the Royal Society of Edinburgh.)

maxillae. Food is collected here and on the long anteriorly directed spines from the basal endites of the maxillule and maxilla, but the latter form the true filtering apparatus, as is shown by the great accumulation of particles on the ventral side of the combs. The maxillae act, therefore, both as a suction-pump and a true filter. The collected food is pushed by the setae on the proximal endites of the maxillules, of the maxillae and of the first trunk limbs, between the bases of the paragnaths on to the middle, spinous region of the mandibles. If there is a great accumulation, this is assisted forwards by the forwardly directed setae on the inner

flanges of the trunk limbs and all the mouth parts become extremely active. The mass is bitten into by the incisor processes of the mandibles and the fragments pushed upwards via the spinous region to the dorsal, molar processes where they are ground up and finally sucked into the oesophagus by peristalsis. Unpalatable matter is thrown out sideways by the mouth parts or, if in great quantity, by violent movements of the whole animal. There are no labral glands and no evidence of any viscid secretion. Food is either filtered directly from the water, or, if there is little live plankton, the animals swim to the bottom where they assume a vertical position, head downwards, and draw up particles from the bottom deposits[1].

Amphipoda. *Ampelisca* is known to feed on fine particles, Hunt (p. 574) having described the process. The animals live in tubes or pockets of mucus to which sand adheres, the mouth being raised slightly above the sea bottom while the lower end is closed. "When feeding the animal lies with its head and its hinder end both near or just protruding from the opening of the tube, the body being strongly flexed into the form of a U, with the back directed downwards. The pleopods are kept in constant motion, driving water out of the tube over the telson. The water driven out is replaced from outside and so a constant current is kept up, entering over the head and mouth parts and directed outwards over the telson. Food particles brought in by the current are seized by the gnathopods and mouth parts, the generally setose character of which is probably useful in straining off and selecting minute particles."

Decapoda. Potts (1915) has shown that the mouth parts (and also the stomach) of the gall crabs, *Hapalocarcinus* and *Cryptochirus*, are greatly modified. They live in chambers within corals and must collect food from the plankton or suspended matter brought in by the water. The buccal area is exceptionally wide and, since the third maxillipeds are set far apart, entirely uncovered. It is screened by a sieve of setae which fringe the endopodites of the third maxillipeds and the exopodites of the first and second maxillipeds. Those parts of the first and second maxillae which are usually concerned with mastication are greatly reduced, while the mandibles, though efficient, are less strongly chitinised than usual, and Potts suggests that they may be used for food sifting or current creating. Both Potts and Hunt (p. 574) state that the crab *Porcellana* feeds on suspended matter, a current, from which food is strained by the strongly setose mouth parts, being produced by the third maxillipeds which make alternate casting movements. *Pinnotheres pisum*, according to Orton (1921), collects mucus-laden strings from the gills of the host Lamellibranch with its setous fringed claws from which they are scraped off by the mouth parts against which the claws are held. Orton (1927) finds that *Eupagurus Bernhardus* seizes its food by its small claw and third maxilliped, the latter being probably alone used during certain periods of the year when the food is largely micro-organisms.

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[1] Cannon (1927) has recently described the feeding mechanism of *Nebalia*, in which a food stream is drawn in *anteriorly* by the oscillatory movements of the foliaceous trunk limbs, particles being retained by a filter formed of setae lining the inner edges of the trunk-limb endopodites. Thence they are brushed on to the gnathobases by a series of feathered setae, and passed to the mouth-parts which closely resemble in structure and function those of Mysids.

Insecta. Clemens (1917, p. 23) states that the nymph of the May-fly, *Chirotenetes*, is specialised for feeding on minute pieces of vegetable matter. When feeding the fine hairs which cover the forelegs and mouth parts meet and overlap to form a straining apparatus which is held against the current. "The elongated fringed labial palpi were extended to sweep in the materials caught, while the maxillary palpi worked laterally and the glossae of the labium working vertically pushed the food materials back to the mandibles." By moving a leg outward, unwanted material was swept away by the current. With the amount of food available, the alimentary canal from the mouth to the end of the mid-gut could be filled about eight times in twelve hours. The larvae of *Culex* and *Simulium* (Naumann, 1924) are passive filtraters, feeding mechanically by means of two large and rapidly moving groups of bristles on the upper lip.

General remarks on fine particle feeders.

Animals possessing feeding mechanisms for dealing with small particles have many things in common. They are all aquatic and many of them sessile. The food is usually entangled with mucus which is found in practically all except the Crustacea, where it is replaced, in the Branchiopoda and Ostracoda at least, by a viscid secretion. There is seldom any but a quantitative selection of particles, feeding being purely mechanical. Mastication is unnecessary, and organs for this purpose are infrequent; similarly the lack of necessity for preliminary digestion has led to the absence of "salivary" glands. Similar food is responsible for the presence of similar feeding mechanisms in distinct groups; thus not only have the ciliary feeding Gastropods developed similar mechanisms to those of the Lamellibranchs, but the lophophore of the Brachiopods has many points in common with the gill of the Mollusca. The former, as Orton (1914) has pointed out, have failed where the Lamellibranchs succeeded probably owing to the absence of fusion of the gill filaments and of the mantle lobes, the inability to form siphons and the lack of organs of locomotion, to which may be added their failure to develop labial palps. The presence of gill-bars of very similar type with lateral cilia for current production and frontal cilia for food transportation has also been emphasised by Orton (*loc. cit.*) and will have been noted in the foregoing account. This group of animals corresponds exactly to Jordan and Hirsch's Whirlers and nearly to Hunt's Suspension Feeders; their food consists of phytoplankton, some of the smaller zooplankton, and suspended organic matter.

II. MECHANISMS FOR DEALING WITH LARGE PARTICLES OR MASSES.

A. For swallowing inactive food.

Under this heading may be grouped a number of animals which swallow, without much discrimination, great quantities of mud, sand and other bottom deposits, from which they extract nourishment as it passes through the alimentary system.

Echinodermata. Amongst the Holothurians, the Aspidochirota shovel bottom deposits glued together with mucus into the mouth by means of their buccal tentacles, while in the Synaptidae food is conveyed inward by the individual move-

ments of the tentacles (Gislén, p. 243). Both live on or in the sea bottom and their stomach contents correspond to the character of the bottom on which they live (Hunt, p. 576). Crozier (1918) has given some indication of the amount of bottom material ingested by Holothurians. The feeding of Spatangids has been investigated by many, notably Robertson (1871), Grave (1902), Eichelbaum (1909), Gandolfi Hornyold (1909, 1910) and Gislén. These animals burrow in sand or mud and maintain communication with the surface by a mucus-lined canal (not always present according to Gislén), through which a respiratory stream is drawn while the rosette feet of the mouth collect particles of sand or food material, the small circumoral spines pushing the food into the gut. Neither Gislén nor Gandolfi Hornyold support the older view of v. Uexküll (1907) that bottom deposits are shovelled into the mouth. Hunt (p. 577) says that the burrowing Ophiuroids, *Amphiura*, *Opiactis* and *Ophiopsila*, all "push bottom material and detritus along the arms to the mouth by means of their tube-feet," with apparently little selection; Blegvad (p. 62) and Gislén (p. 254) make similar statements regarding *Amphiura*.

Annelida. Many Polychaetes, such as *Arenicola*, *Aricia*, *Scalibregma*, *Ammotrypane*, *Notomastus* and members of the Maldanidae (Hunt, p. 577, Blegvad, p. 60) swallow bottom deposits by the aid of their soft, extensible probosces with little or no attempt at selection. They bury themselves in the bottom and where, as in *Arenicola*, they are uncovered at low tide the proboscis may be seen at the bottom of a funnel-shaped depression "moving up and down swallowing the matter drawn down from the sides and circumference" (Blegvad). Flattely (1916) describes the feeding of *Cirratulus tentaculatus* which, though it burrows in mud or sand, only contains algal spores, fragments of decaying algae, diatoms and other organic matter in the gut which is ciliated throughout. The tentacles are not used in feeding, the process apparently consisting of a kind of suction, selection being exercised by sensory flaps on the walls of the pharynx which prevent any but the smallest and most suitable particles from entering the gut. Among the Oligochaetes, the earthworms take in earth with the included organic matter largely by means of the sucking action of the pharynx (Jordan, 1913, p. 192). Sipunculids swallow mud and sand with their muscular introverts.

Mollusca and Crustacea. Since Hunt (pp. 580-1) found roughly sorted bottom material in the stomachs of the Gastropods, *Turritella communis* and *Aporrhais pes-pelicani*, and also in the burrowing Decapods, *Callianassa subterranea* and *Gebia stellata*, it appears that the feeding mechanisms of these animals must be concerned with the swallowing of such food.

General remarks.

Animals feeding in this manner are usually sluggish and frequently burrow. They exercise little, if any, selection, swallowing all that lies before them by means of extroversible gullets, pushing tentacles and similar mechanisms. Organs of mastication and of preliminary digestion are of little use and are seldom found. They are omnivorous and correspond closely to Hunt's deposit feeders; they are included under Jordan and Hirsch's Snarers—not at all a good description.

B. *For scraping and boring.*

Mechanisms of this type are possessed by a somewhat miscellaneous collection of animals which feed either by boring their way into hard substances, the fragments being swallowed and digested, or by scraping off encrusting animals or plants, or else eating dead, or slowly moving animals by boring into them, scraping off and swallowing the flesh in the process.

Echinodermata. *Echinus* and closely related genera which live on rocks feed on the encrusting organisms which they tear off and masticate with their five strong teeth, and also on bottom material which is conveyed to the mouth by the tube feet. According to Krumbach (1914), the species found near the coast are mainly plant feeders while those which have migrated into deeper water are predatory.

Mollusca. The radula of the Ampineura and the Gastropoda is used chiefly for scraping food into the mouth, often with the aid of jaws, and for boring into animal prey. The foot is used to provide a firm purchase for the working of the radula. The radula ribbon is secreted in a special caecum and is applied to the surface of paired cartilages on the floor of the buccal cavity. The mechanism of this odontophore apparatus has been the subject of a great deal of work which has been ably summarised by Amaudrut (1898) and Herrick (1906). One group of workers, chief among whom are Huxley (1853), Wegmann (1884), Oswald (1893), Herrick (1906) and Dakin (1912) consider that the radula is drawn backwards and forwards over the cartilage as over a pulley; while another, the principal protagonists of which are Geddes (1879), Amaudrut (1898) and Simroth (1901 *b*), consider that the rasping movements are dependent on those of the supporting cartilage.

The odontophore is brought into play or withdrawn into the buccal cavity by the action respectively of protractor and retractor muscles attached to the cartilages. In many of the carnivorous species it is carried on the end of a long, extrusible proboscis and the mechanism in one of these, *Sycotypus*, the anatomy of which is shown in Fig. 20, has been described by Herrick (1906). The cartilage acts as a supporting framework and as a grooved pathway in which the radula (*r.*) and its great retractor (*g.*) work. (In the figure the rami (*cr.*) have been separated, but in life they lie close together and, since their inner surfaces are grooved, enclose the great retractor.) The rami are bound together by muscles (*vsf.*) which ensheath the great retractor, and the whole cartilage is protracted by a series of narrow muscles (*pc.*) which arise from the outer margins of the rami and are inserted, more anteriorly, in the ventral wall of the proboscis, and by a pair of small triangular protractors (*tc.*) one on either side of the anterior region of the buccal mass. Retraction is performed by two flat muscles (*rc.*) arising from the posterior ends of the rami and inserted in the base of the proboscis. The movements of the radula, *i.e.* the actual rasping of the food, are controlled by six long protractors situated on the ventral side, three on either side of the middle line (*pr. (a, b, c)*), which arise from the membranous pouch in which the anterior and

mature part of the radula lies and pass backwards into the base of the proboscis; and by the powerful great retractor (*g.*) which is inserted by tendons into the anterior region of the radula sac, in which the radula is secreted and in which the more posterior, immature parts lie, and passes backwards as a single muscle with four separate roots attached to the wall of the proboscis and on to the rami. The radula sac (*rs.*) continues for some distance posterior to the insertion of the great retractor, finally merging into the radula muscle (*rms.*). Since the teeth of the radula are inclined backwards, the effective action is on the return pull which explains the greater size of the retractor. The radula ribbon "is folded together as it passes over the cartilage, and unfolded as it passes forward; otherwise the animal would rasp its own tissues...the lateral teeth are turned in against each other, so that as the lingual ribbon passes back from the head of the cartilage they are thrown out of action" (p. 721). The proboscis is retracted by its longitudinal muscles and especial retractors, while protraction is probably due to pressure in the head fold consequent on muscular contraction.

The mechanism in Gastropods without probosces, such as *Helix*, is essentially similar, the odontophore being protracted and retracted in the same manner, although, according to Amaudrut, the radula in *Helix* does not slide over the cartilage but is attached to it and moves as it moves; he considers that the muscles described by other workers as protractors and retractors of the radula are tensors which draw the radula tightly against the cartilage during activity. The number and form of the teeth in the radula varies greatly; but, generally speaking, in the carnivorous species the teeth are few and powerful while in the herbivorous Gastropods they are smaller and more numerous.

Fig. 20. *Sycotypus canaliculatus*, ventral view of odontophore muscles, some of which have been cut through; entire wall of proboscis removed except where protractors of cartilage inserted; whole buccal mass pinned out to display the muscles, dorsal sheet of fibres having been cut through, only muscles on right drawn completely. *cf.* cross fibres uniting triangular protractors; *cr.* rami of cartilage; *dsf.* dorsal sheet of fibres cut through; *g.* roots of great retractor; *pc.* protractors of cartilage; *pr.* (*a, b, c*), protractors of radula; *pw.* wall of proboscis; *r.* radula; *rc.* retractor of cartilage; *rms.* radula muscle; *rs.* radula sac; *tc.* triangular protractor of cartilage; *vsf.* ventral sheet of cross fibres. (From Herrick (1906).)

Amongst the browsing Mollusca may be mentioned *Chiton* and also *Patella* which, according to Davis and Fleure (1903), usually scrapes off encrusting algae and other minute organisms but may also seize pieces of seaweed with its outer lips and the palate and then scrape off fragments with the radula; the same movement which causes the tip of the radula to scrape off food causes the more posterior

region to act against the jaw, or palatal plate, which assists in the grinding-up of the food, and this chewing action continues after actual feeding has stopped. Stephenson (1924) reports similar feeding habits in *Haliotis*. The Opisthobranch, *Aplysia*, browses on green algae, largely *Ulva*, which it seizes with the protruding lips and the jaws and rasps with the radula which works against the jaws. In the Nudibranchs it is stated by Alder and Hancock (1865, p. 12) that "the radula is more of a prehensile than a rasping organ." Many of them browse on sessile animals: *Eolis* feeds on anemones, the pieces bitten off by the protruding jaws being seized and passed back by the recurved spines on the radula; *Doris* browses on sponges, the scoop-formed radula being flattened against the food the surface of which is licked backwards into the mouth; in *Tritonia*, the main food of which is *Alcyonium* (Hunt, p. 590), the radula is assisted by a pair of powerful jaws. The herbivorous Pulmonates, such as *Helix*, browse on vegetation.

Many carnivorous Gastropods bore into their prey, using their muscular introverts. Some only bore into flesh; thus Colton (1908) has shown that *Sycotypus* and *Fulgur* (and probably *Nassa* and *Lunatia*) never bore through the shells of Lamellibranchs. *Sycotypus* crawls on to an oyster, waits until it opens its shell valves and then thrusts its own shell between them and pushes its proboscis into the soft parts within. It feeds on *Mya* by pushing the proboscis through the gape. *Fulgur* seizes the strong shell of *Venus* in the hollow of its foot and brings it against the margin of its own shell and by contractions of the columellar muscle forces the valves together so that fragments are broken off, the process being repeated until the crack is about 3 mm. wide. The proboscis may then be flattened and forced in, or the animal inside be killed with a secretion, or the shell may be forced open. François (1891) states that *Murex fortispinna* has a special marginal tooth in the aperture of its shell used for opening the shells of *Arca*. Dakin (p. 104) observed *Buccinum* feed in the same manner on *Pecten*, and also states that the carapace of a crustacean, such as *Nephrops*, can be bored through by the radula. The writer has seen *Buccinum* attack dead squid, rasping off and swallowing large pieces.

Other Gastropods bore through the shells of Lamellibranchs by mechanical or chemical means. In the former case the radula is employed, the evenly worn teeth of which indicate the manner in which they have been employed, as shown by Colton for *Urosalphinx* and *Purpura*. *Murex* also has been observed to bore in this manner. *Natica* has a disc-shaped glandular organ on the under side of the proboscis which, according to Schiemenz (1891), produces an acid secretion for dissolving the shell, while the "salivary" secretion of species of *Dolium*, *Tritonium*, *Cassis*, *Cassidaria* and *Murex* and a number of Opisthobranchs contains varying amounts of free sulphuric and hydrochloric acids which apparently serve for boring into or dissolving calcareous prey (see Semon, 1889, and Jordan, 1913 for full details).

The Teredinidae, or Shipworms, obtain a large part of their food from the wood into which they bore, as shown by Harington (1921), Potts (1923) and Dore and Miller (1923). Miller (1924) has described the method of boring, which is illustrated in Fig. 21. The greatly modified shell valves are held firmly against the head of the burrow by the small, sucker-like foot (*f.*) below and a distended fold

of the mantle (*d.*) above. Rasping is carried out by the anterior lobes (*a.l.*) of the shell which are transversely ridged and are drawn apart, while pressed against the wood, by the contraction of the large posterior adductor which runs between the posterior, auricular lobes (*p.l.*) of the shell. As soon as this movement is completed, the foot is released and takes up a new position—for the anterior end of the body twists continuously in one direction and then the other through an arc of 90°—while the small anterior adductor contracts and draws the anterior lobes together ready for the next boring movement. The wood fragments are probably passed into the mouth by ciliary action as stated by Lazier (1924)[1].

Crustacea. Scrapers and borers are rare, *Limnoria* and *Chelura*, though they bore into wood with their mandibles and pass the fragments into the alimentary system, do not appear to extract nourishment from it (Yonge, 1927). According to Farkas (1923) and Naumann (1923), *Cyclops* and *Heterope* may scrape food which is then broken up in the "atrium" or mouth cavity by the mouth parts and carried
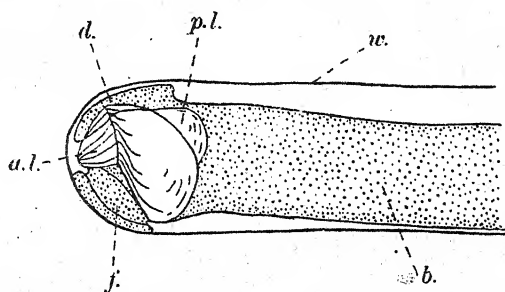
Fig. 21. Boring position of Teredo in end of burrow. *a.l.* anterior lobe of left shell valve; *b.* body; *d.* dorsal fold of mantle; *f.* foot; *p.l.* posterior lobe of shell; *w.* wall of burrow. (From Miller (1924).)

to the mouth by the "rotating organ." Willer (1922) states that *Gammarus* holds on to leaves by the gnathopods and the exopodites of the maxillipeds and tears off pieces with the endites and exopodites of the maxillipeds, crushing them with the maxillae and the mandibles.

Insecta. Only a few examples of scraping or burrowing insects can be given here. Jordan (1911) has shown that the silkworm caterpillar feeds on leaves by holding them fast with its feet, and upper and lower lips, while it bites pieces out with the mandibles. The Termites eat into wood with their powerful mandibles, the swallowed fragments being later digested for them by intestinal flagellates (Cleveland, 1924), and the larvae of *Cetonia* (Rosechafers) have been shown by Werner (1926) to feed on pine needles, the bitten fragments of which are digested with the help of bacteria in the enlarged mid-gut.

General remarks.

Animals which feed by scraping and boring are all slowly moving and possess powerful buccal armature—radula, jaws, teeth, modified shell valves, mandibles,

[1] The Teredinidae also possess reduced gills and small palps by means of which a certain amount of plankton is collected and passed into the mouth.

etc. They also possess organs for holding on to the food mass, such as tube feet, appendages or foot. They cannot swallow food until it is much reduced, and so feeding is a slow, and usually a continuous, process. Most of them are selective feeders, though there are exceptions, notably *Echinus*. In some cases boring is assisted chemically, as in the carnivorous Gastropods. Preliminary digestion, except in the Gastropods, is usually slight. Animals with this type of feeding mechanism correspond to the scrapers of Hirsch, and Jordan and Hirsch.

C. *For seizing prey.*

Included under this heading are the feeding mechanisms of the majority of carnivores which seize and devour living prey.

(i) *For seizing only.*

Protozoa. Many of these have feeding mechanisms of this type, three examples of which are given here. In the words of Schaeffer (1916) Amoebae are "essentially beasts of prey," for they devour Protozoa of any size from small Flagellates to *Paramaceium*, also Rotifers and small Entomostraca as well as Diatoms and Desmids. It is only essential that they should be slowly moving so as to allow of the formation of a food cup over them. Among the Ciliata especially, carnivorous species are common, of which the most striking are predatory animals like *Didinium nasutum*, which buries its proboscis, armed with a seizing organ of dense protoplasm, into the body of its victim—usually another Ciliate—which it swallows whole (Calkins, 1915). Maupas (1885) states that *Coleps hirtus* seizes other ciliates, such as *Glaucoma pyriformis*, by means of the peribuccal denticles and swallows them whole.

Coelenterata. These are all carnivorous and, with the exception of those with ciliary feeding currents, usually seize and paralyse their prey with their tentacles armed with nematocysts. The feeding of *Hydra* has been most recently described by Goetsch (1921) and Beutler (1924), and that of the hydroids by the latter (1926), who has shown that the individual polyps do the same work as the solitary *Hydra* in capturing and digesting the prey, usually small crustaceans, the constriction at the entrance to the stalk preventing any but the smallest particles or matter in solution from passing into the branches and common stem of the colony, in the endoderm of which absorption takes place. Lebour (1922, 1923) has described in detail the food and feeding of a variety of medusae, voracious creatures which often swallow prey larger than themselves, seizing it with the tentacles, and in some cases with the manubrium, and then passing it into the mouth. Fig. 22 gives some indication of the possible size of the prey. The same author has also described the feeding of young Scyphozoa; the ephyrea of *Aurelia* stings young fish with the edge of the lappel and then envelopes them with the umbrella until they are fixed in the manubrium, while the small adults use the marginal tentacles for capture and the long lips for conveyance into the manubrium. Gemmill (1920) observed ephyrea capturing Ciliates with its arms and then passing them to the mouth. Young *Chrysaora* collect food, usually Coelenterates and *Sagitta*, with the tentacles which then contract, the lips sweeping off the food which collects in a

temporary bag formed by them beneath the stomach. The feeding of Actinians has been described by many; the majority capture food with tentacles armed with nematocysts, as described by Carlgren (1905) for *Tealia*. Parker (1917) has discussed the subject in detail. The tentacles of *Isophyllia* (Fig. 23, *T.*), the rose coral, as shown by Carpenter (1910), seize with their knob-like distal ends all objects which touch them. After securing plankton, the oral disc (*O.D.*) sinks and the marginal zone (*E.Z.*) folds inwards, finally roofing in the tentacles and oral disc and forming a superficial chamber (*S.C.*), into which the stomadaeum (*St.*) and
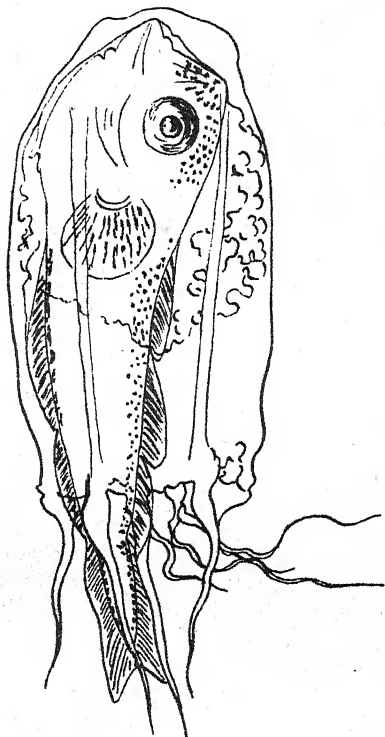
Fig. 22. *Turris pileata*, 25 mm. long, containing young Whiting. (After Lebour (1923), Fig. 8. By kind permission of the Marine Biological Association.)
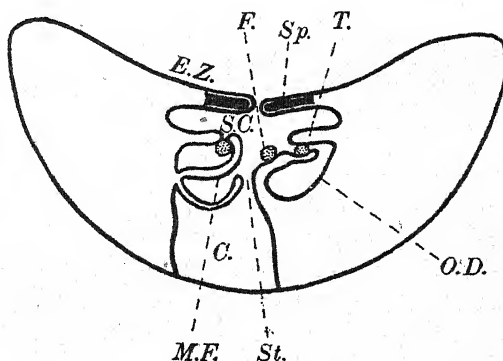
Fig. 23. *Isophyllia*, diagrammatic vertical section through a contracted feeding polyp. *C.* coelenteron; *E.Z.* edge zone, folded over; *F.* food particle; *M.F.* mesenterial filament, extruded; *O.D.* oral disc; *Sp.* sphincter muscle, contracted; *St.* stomadaeum, everted; *S.C.* supra-discal cavity; *T.* tentacle. (From Carpenter (1910).)

mesenterial filaments (*M.F.*) project, and where they probably digest and absorb the prey. Boschma (1925) has reviewed the literature on feeding in corals, and shown that *Astrangia* will capture small Copepods with its tentacles after which the mouth with the central part of the disc forms a conical protuberance which moves towards the prey, the tentacles at the same time bending downwards; when the two meet the prey is released and falls through the stomadaeum into the gastric cavity. Pratt (1906) finds that *Alcyonium* selects its prey with its tentacles, plankton being chiefly taken. Lebour (1923) has described the feeding of the Ctenophore,

Pleurobrachia, which captures passing prey with its tentacles which immediately contract and convey it to the mouth.

Turbellaria. These animals feed by means of a muscular pharynx which can be extended to about half the length of the body, a process which has been described by, amongst others, Pearl (1903), Westblad (1923) and Hyman, etc. (1924). Under laboratory conditions they can be fed on soft meat, but their natural food, according to Westblad, consists of Protozoa, Nematodes, and small Crustacea such as *Asellus*, in the case of Triclads, and of Rotifers, small Crustacea, small Oligochaetes and sometimes Algae, in that of Rhabdocoels. In Triclads, where digestion is purely intracellular, there is no preliminary digestion, but in certain of the Rhabdocoels where the gut epithelium is well-defined and not amoeboid, a digestive secretion from pharyngeal glands is poured over the food as it passes into the gut (Westblad).

Echinodermata. Many Ophiuroids, such as *Ophiura* and *Ophiocoma* (Hunt, p. 587) and *Ophioglypha* (Blegvad, p. 62), feed on living Polychaetes, small Crustacea or Molluscs, seizing them with surprising agility by means of their mobile arms which enwrap the prey and carry it to the mouth where it is swallowed whole. Many Asteroids feed on similar prey, capturing it with their tube feet and swallowing it whole; thus *Luidia* may take in complete *Spatangus* (Hunt, p. 586), but, since they frequently digest it externally, they will be considered later.

Nemertea. These are all carnivores, the larger species feeding on worms and the smaller ones on Crustacea. *Lineus longissimus* has been observed eating gobies and *Lumbriconereis* (Hunt, p. 583), which, since the feeding mechanism consists of a muscular introvert with small stylets in the case of the armed species, must be swallowed whole.

Annelida. Many Polychaetes, such as *Aphrodite*, *Nephthys*, *Glycera*, *Lumbriconereis*, etc. (Hunt, p. 588, Blegvad, p. 68), seize living prey, usually other worms, crustaceans, or molluscs, with their muscular introverts armed with chitinous teeth, no mastication takes place, the prey being swallowed whole to be digested within, perhaps first crushed up as in the muscular pharynx of *Aphrodite* (Jordan, 1904 *b*). The feeding of *Nereis virens* has been studied by Gross (1921) and Copeland and Wieman (1924); though usually carnivorous, it may also eat Algae. Lebour (1923) states that *Tomopterus* may swallow entire *Sagitta* or larval herrings. The aquatic Oligochaetes, *Nais* and *Stylaria*, are stated by Cori (1923) to capture living prey by everting the pharynx.

Gephyrea. *Priapulus* and *Halicryptus* are classed as "carrion detritus eaters" by Blegvad (p. 69) who found remains of Polychaetes within them. They possess a muscular introvert, but little is known of their mode of feeding.

Chaetognatha. *Sagitta* and *Spadella* possess, on either side of the mouth, a series of sickle-shaped chitinous hooks which work horizontally and with which, according to Lebour (1923), *Sagitta* seizes its prey at any part of the body, usually swallowing it whole. Young fish, other *Sagitta* and Copepods are usually eaten.

Rotifera. In *Stephanoceros* (Ubisch, 1926), prey, such as *Euglena*, is captured by means of the five long arms fringed with extremely long cilia which entrap the food and hold it till it is swallowed.

Mollusca. Although the majority of the Gastropoda are scrapers or borers, a few, usually either without, or with modified, radulae, swallow their prey whole. Among the Tectibranchs, the Scaphandridae, Bullidae and Aceratidae and also *Philine* swallow small Lamellibranchs whole crushing them to pieces with the calcareous or chitinous plates lining their muscular gizzards (see Hirsch, 1915, and Hunt, p. 590). Other examples are found in the Nudibranchs. *Tethys* (Krumbach, 1917), which has neither radula nor jaws, captures its prey, usually young fish, by means of two large head lobes which act as a kind of ladle; if accepted by the mouth it is swallowed whole. *Calma glaucoides* feeds on the eggs or embryos of the smaller, shore fishes, as described by Evans (1922). During feeding the face of the animal fits like a hood over the egg which is slit open by the narrow, saw-like radula, the contents being swallowed with the aid of the lateral jaws. Eliot (1910) states that in *Melibe*, which has no radula and only feeble jaws, a deep funnel with contractile margins and long cirri surrounds the mouth, which sweeps over the surface of stones capturing as in a net small, or even relatively large, Crustacea. The funnel is then tossed up, the margin contracted, the opening closed with the cirri, and the prey drawn down into the stomach where it is crushed up by the girdle of plates. Certain of the slugs, such as *Testacella* and *Daudebardia*, which have exceptionally large and powerful pharynxes, swallow whole earthworms or snails, which they draw out of their shells, holding them fast with the long teeth of the radula.

The Septibranchs are the only carnivorous Lamellibranchs. The structure and function of the feeding organs of *Cuspidaria* and *Poromya* have been studied by Yonge (1928). They live in mud and draw in small Crustacea or Annelida, either slowly moving or dead, through the inhalent siphon as a result of movements of the muscular septum. Normally the septum lies quiet with open pores through which a slight current is maintained by the fringing cilia from the infra- to the supra-septal cavity, but several times a minute the septum is lowered slowly, the pores are closed and then a sudden upward movement takes place causing water and food to be drawn in quickly through the inhalent, and water as violently ejected through the exhalent, siphon. The food is retained in the infra-septal cavity by a large valve which guards the opening from the inhalent siphon, and is pushed into the mouth by the small but very muscular palps. Cilia are greatly reduced and are only concerned with the removal of fine particles from the mantle cavity.

(ii) *For seizing and masticating.*

Here are considered feeding mechanisms which serve not only to capture prey, but also, to a greater or less extent, to masticate or tear it.

Mollusca. The feeding mechanisms of a number of Gastropods are most suitably considered here. Hirsch (1915) states that *Pleurobranchia* lives in mud and preys on carrion, seizing it with its muscular proboscis and rasping the tissues with its powerful radula. The Heteropods, *Pterotrachea* and *Carinaria*, feed on small animals of the plankton in a somewhat similar manner. They have no jaws but a large protractile pharynx and a radula with powerful lateral and marginal

teeth. The Gymnosomatous Pteropods are voracious carnivores feeding largely on Thecosomatous Pteropods. They have usually an evaginable proboscis generally bearing buccal appendages for seizing prey, such as hook-sacs—a pair of evaginable sacs on either side of the radula bearing recurved hooks—and, in the Pneumono-dermatidae, suckers. In the Clionidae and in *Thalassopterus* the short pharynx bears paired appendages known as "cephalocones" which secrete a sticky fluid which entangles the prey. There are jaws and powerful radulas with 20–30 teeth in each row. The feeding of these Pteropods has never been properly studied, but full details as to their feeding mechanisms and references to literature are given by Tesch (1913, p. 94). Unlike scrapers, none of the above have holding organs.

Crustacea. These are largely omnivorous but many feed chiefly on living prey or carrion, which is torn up by the mouth parts before being passed on to the gastric mill for complete trituration; the latter process cannot be discussed here but details are given by Jordan (1904 *a*) for *Astacus* and Yonge (1924) for *Nephrops*. Lundblad (1920) states that *Lepidurus* feeds on small animals such as Ostracods, seizing them with the appendages near the mouth and masticating them with the powerful mouth parts. In Mysids, as shown by Depdolla (1923) and Cannon and Manton (1927), food such as small Crustacea and *Sagitta* is held by the thoracic endopodites, adjusted over the mouth parts by the mandibular palps and then bitten into by the incisor processes of the mandibles aided by the distal endite of the maxillules[1]. Isopods are often carnivorous; Blegvad (p. 71) states that *Idothea* may attack young fish, while Tait (1927) has described the feeding of *Chiridotea*, which lives on carrion, seizing it with its three pairs of gnathopods which manipulate it and feed it to the "mandibular mill," where it is pushed forward about once a second and more slowly withdrawn, pieces being removed by the mandibles, which work about four times more rapidly, at each operation and passed into the oeso-phagus. The action of the mandibles is essentially biting rather than chewing. The food of the Decapods, largely flesh, is described by Hunt (p. 591) and Blegvad (p. 70). Borradaile (1917) states that in *Leander* small pieces are conveyed directly by the chelipeds to the second maxillipeds which usually hold them while fragments are torn off by the deeper-lying mouth parts, especially the mandibles. Larger pieces are handled by the chelipeds and third maxillipeds until they can be seized by the indispensable second maxillipeds. The incisor processes of the mandibles tuck the food into the lip-chamber, but there is probably some tearing, while before the food enters the oesophagus it is pounded by the molar processes. According to Herrick (1895), in *Homarus americanus* the food is held against the mouth parts by the third maxillipeds and, by means of the cutting spines on the maxillae and first and second maxillipeds, "the meat is as finely divided as in a sausage machine, and a stream of fine particles is passed constantly into the mouth, being previously submitted to the action of the mandibles" (p. 32). This is hardly in accordance with other observations on Decapods. In *Nephrops* (Yonge, 1924) and *Palinurus* (personal observations) the food is held by the mandibles while it is shredded by

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[1] *Nebalia* (Cannon, 1927) feeds on large particles in a similar manner, but it cannot directly pick up large particles; preliminary mastication is carried out exclusively by the maxillules, the mandibles having no biting incisor processes and being symmetrical.

the action of the third maxillipeds, being cut up when it can be swallowed, the large pieces in the stomach indicating the slightness of mastication. Again, in *Carcinus maenas*, Borradaile (1922) states that food seized by the chelae is placed between the mandibles which do not cut or chew but hold it firmly while it is being divided by the other mouth parts unless it is extremely soft when it may be pushed directly into the mouth by the mandibular palps and the nose of the labrum. Usually it is torn by being pulled outward by the chelae or, more often, the third maxillipeds which, as in *Nephrops* and *Palinurus*, grasp it with the toothed inner edges of their ischiopods. The second maxillipeds may assist or replace them, or cut the food between the mandibles and the third maxillipeds; the first maxillipeds probably guide food to the mouth; the maxillae are unimportant but the maxillules cut the food with their outer, and push it into the mouth with the inner, laciniae.

Insecta. Here are many active carnivores, such as the Mantids which seize living insects with their sub-chelate anterior legs; the Libelludidae, the aquatic larvae of which capture prey with the "mask"—modified second maxillae and labium (Miall, 1912), the adults feeding on the wing by means of the large mouth closed by mobile lips; and the Cicindelidae, or Tiger-beetles, which capture prey with their powerful mandibles, squeezing them in the large mouth which forms a press, being closed by the labrum above and the palpi laterally.

Arachnida. The Scorpions are predaceous carnivores, seizing insects and spiders with their chelate pedipalps and breaking them up with the chelicerae. Large prey is paralysed with the tail sting. The Phalangidae, or "Harvestmen," feed on mites, myriapods, insect larvae or spiders. Kästner (1925) states that in *Mitopus*, *Phalangium* and *Platybunus*, the chelicerae break up the food, the fragments of which are packed behind the mouth and later pushed in by the base of the first legs and the labium, the base of the pedipalps probably preventing food from escaping anteriorly.

Myriapoda. Plateau (1876) has described the feeding of *Lithobius forficatus* which rapidly devours insects (disposing of a fly in five minutes), seizing them with the poison claws which it forces well into the body. The anterior part of the body is then erected at an angle of 45° so that the prey can be held, abdomen upwards, to the mouth, against which it is directed by the first maxillae and where it is quickly masticated by the powerful mandibles, which, aided by the two pairs of maxillae, push the fragments into the mouth. When the harder thorax is reached, the contained muscle is dug out and the empty chitin abandoned.

(iii) *For seizing followed by external digestion.*

In certain animals food is largely digested external to the gut, the soluble products being sucked in or absorbed by extruded regions of the gut. Jordan (1910) and Lengerken (1924) have reviewed and added to the literature on the subject.

Protozoa. Cienkowski (1865) described how *Colpodelia* and *Vampyrella*, members of the Sarcodina, feed on freshwater algae, eating through the cellulose wall by means of an extruded cellulase.

Echinodermata. The Asteroids are largely predaceous carnivores or carrion

feeders. Schiemenz (1896) divided them into those, like *Astropecten*, with pointed tube feet, which live in sand and swallow small Lamellibranchs, and those, like *Asterias*, with sucker tube feet, which can crawl on rocks and feed on larger bivalves which they pull open. Blegvad (p. 63) thinks that poison is used as well as suction in this process. After seizure, if too large to be swallowed whole (and there are no masticatory organs), prey is digested externally, the stomach being extruded and the prey digested by the enzymes secreted. Schiemenz observed two *Asterias* devour an *Echinus* by forcing their stomachs down its mouth, the author has watched them attack the Decapod, *Munida*, dissolving out the tissues and discarding the empty shell.

Mollusca. Many of the carnivorous Gastropods probably extrude protease from their "salivary" glands over their food and so assist the mechanical action of the radula in breaking it up. In the Cephalopods, such as *Sepia* (see Fig. 24), food is captured by arms and tentacles armed with suckers and held by them against the buccal mass which is surrounded by a circular lip (*l.*) and armed with a pair of powerful chitinous jaws, one ventral (*v.j.*) and one dorsal (*d.j.*), the tip of the former

overhanging the latter, together with a radula (*r.*) on the floor of the buccal cavity. To the jaws are attached the powerful muscles which form the bulk of the buccal mass, while the radula, as in the Gastropods, is worked by protractor and retractor muscles. Anterior and ventral to the radula is the sub-radular organ (*s.r.*), while the radula is enclosed by a pair of outgrowths from the dorsal wall of the pharynx, the "Zungentasche" (*z.*). There is a complex series of "salivary" glands, varying somewhat in different groups but consisting typically of a pair of large posterior glands opening by a common duct (*p.s.*) at the tip of the sub-radular organ, a much smaller anterior pair (*a.s.*) which open by separate ducts into the sides of the pharynx and a small sub-lingual gland, an infolding of the epithelium in front of the sub-radular organ.

The process of feeding is difficult to follow and much work remains to be done. The food is bitten into by the jaws and rasped by the radula against which it is held by the "Zungentasche," but of primary importance is the secretion of the posterior "salivary" glands. This contains mucus, a poison allied to tyramine (parahydroxyphenylethylamine)
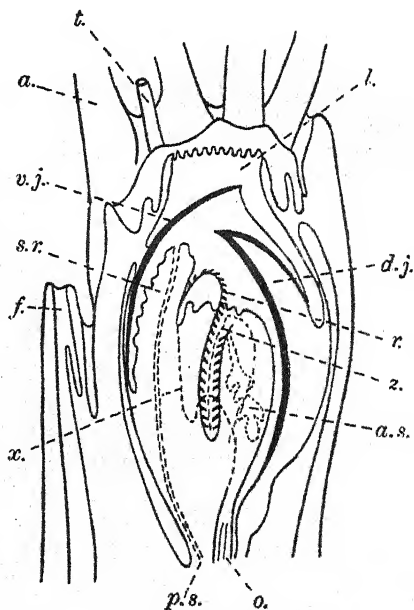
Fig. 24. Sepia, diagrammatic representation of feeding organs. *a.* arm, with suckers to base; *a.s.* anterior "salivary" glands; *d.j.* dorsal jaw; *f.* funnel; *l.* lip; *o.* oesophagus; *p.s.* duct of posterior "salivary" glands; *r.* radula; *s.r.* sub-radular organ; *t.* tentacle, long with suckers near end; *v.j.* ventral jaw; *x.* ventral boundary of pharynx; *z.* "Zungentasche." (Adapted from Blochmann and Hamburger (1924), *Vorlesungen über Vergleichende Anatomie*, Lief. 4, Fig. 111.)

according to Henze (1905), and also a strong protease working best in alkaline media (see Krause, 1895, 1897 and Falloise, 1906) by means of which the living prey—usually fish or Decapod Crustacea—is killed and digested. The shell of Crustacea is opened, probably with the jaws, and the flesh within digested with injected secretion, the fluid is then sucked into the oesophagus and the intact, but empty, shell rejected. The flesh of fish is dissolved away and the bones discarded in the same way. Comparatively large pieces of food may be swallowed, Scott (1910) reporting the presence of remains of Crustacea in the gizzard of *Stauroteuthis hippocrepium*, and the importance of external digestion probably varies in different groups, a problem which needs further investigation. The function of the other "salivary" glands is obscure.

Insecta. External digestion is especially widespread here; only a few examples can be cited but further details are given by Lengerken (1924). The larvae of many Diptera feed in this manner, muscid grubs liquefy flesh by ejecting protease over it, the aquatic "Phantom Larva" of *Corethra* captures small animals by its antennae, crushes them with its mandibles and presses them into the mouth, the back of which is closed by a fringe of bristles, where they are digested externally (Miall, 1912). Springer (1917) states that the larvae of *Miastor* live in groups on wood which they dissolve away with enzymes till they come to lie in grooves bathed in a nutrient fluid which they easily suck in. The ectoparasitic hymenopteran *Pseudogenia* is stated by Ramme (1920) to feed on spiders pouring over them a secretion which digests them externally, even to the chitin, in 48 hours. In all the above instances, the enzymes are produced by the "salivary" glands. Among the Neuroptera, the larva of *Myrmeleon*, the Ant-lion, which lives at the bottom of conical pits it constructs in the sand, captures with its elongate mandibles ants and flies which fall into the pits, killing them with poison in from 1–30 minutes (Stäger, 1925). The flesh is digested externally and the fluid sucked by the action of the muscular pharynx into the buccal cavity through grooves along the sides of the mandibles (Sharp, 1895, p. 455).

In the Coleoptera, Jordan (1910) and Lengerken (1924) have shown that the larva and imago of *Carabus*, which are carnivorous, when about to feed vomit a digestive and toxic secretion from the mid-gut and then work on the half digested flesh with the mandibles. Heymons and Lengerken (1926) find the same condition in *Silpha*. The larva of *Dytiscus* is probably the most highly specialised of external digestors. The mouth is excessively small but each of the very long and needle-like mandibles possesses a longitudinal canal. When the mandibles are extended their basal openings are outside the mouth which is open, but when they close on a victim the basal openings are brought within the corner of the mouth which is closed by means of a grooved "mouth lock." Poison and digestive enzymes secreted by crypts of the mid-gut are then forced into the prey by way of the mandibular canals and, after the flesh has been dissolved, it is sucked back by the pumping action of the pharynx through the canals into the gut (Miall, p. 45, Rungius, 1911). A caddis larva may be digested in 10 minutes. In very similar manner, poison and digestive enzymes are forced into the prey through the mandibular canals of the

larva of *Lampyris* (Vogel, 1915) though the mouth may assist in the process. The process of softening is assisted by the teeth on the mandibles, the digested mass being finally pushed into the mouth by the maxillae and the hairs on the mandibles. Living snails and slugs are disposed of in this manner.

Arachnida. The Araneae or true spiders capture their insect prey either by means of webs or by chasing them in the open. The fangs of the chelicerae are driven into the body of the victim which is killed by poison from their glands. A protease is then injected, probably from the pedipalp glands (Bertkau, 1884, 1885), liquefying the flesh which is then sucked in through the mouth by the action of the muscular pharynx, the empty chitin being abandoned.

General remarks.

Feeding mechanisms for the seizing of prey are characterised by the presence of special seizing organs, such as tentacles with nematocysts or suckers, jaws, radulae, chelae, chelicerae, hooks, muscular protractible pharynxes, etc., and not holding organs as in the scrapers and borers. The prey is often poisoned, by nematocysts, the stomach secretion of Asteroids, or by the poison glands of carnivorous Gastropods, Cephalopods, Insect larvae, Spiders and Myriapods. "Salivary" glands for preliminary digestion are common. Animals possessing feeding mechanisms of type (i) are usually sessile or slowly moving, seizing their prey as it passes, but animals with mechanisms of types (ii) and (iii) are usually active and predaceous. Food is usually disposed of very quickly—unlike the scrapers and borers—and feeding takes place irregularly, as much as possible being consumed in any given time. Food is swallowed whole by animals with type (i) mechanisms, and often only slightly broken up by animals with type (ii) mechanisms where it is usually further broken up in the alimentary canal by the aid of a muscular pharynx, as in *Aphrodite*, a triturating gizzard or stomach with chitinous or calcarate armature, as in *Scaphander* and its allies, the Septibranchs, Decapod Crustacea, many Insecta, etc. Where there is no mechanical means of trituration, food is broken down directly by means of proteases, as in the Coelenterates and Echinoderms, and external to the gut in the animals with mechanisms of type (iii). The animals with feeding mechanisms of this nature correspond to the snarers of Jordan and Hirsch and the carnivores of Hunt.

III. MECHANISMS FOR TAKING IN FLUID OR SOFT TISSUES.

(i) *For piercing and sucking.*

Under this heading are considered animals which first pierce and then suck, a few are vegetable feeders but the majority are parasitic.

Nematoda. *Ancylostoma duodenale* holds on to and pierces the epithelium of the gut with its chitinous teeth and sucks the tissues by means of its pharynx.

Annelida. Eisig (1906) has described the feeding of the Polychaete *Ichthyotomus sanguinarius* (see Fig. 25) which sucks the blood of the fish, *Myrus vulgaris*, cutting the flesh with its shear-like stylets (*v.s.*, *d.s.*) and pumping in the blood with its

pharynx (*a.p.*). An anticoagulin is secreted by glands opening near the mouth (*g.'*, *g."*). The leeches may all be considered here, although only one section possesses piercing jaws, usually three, the other having a proboscis. They all have suckers, "salivary" glands for producing the anticoagulin, hirudine, and a sucking pharynx.

Crustacea. The parasitic Copepods feed on the body mucus or blood of the host fish. Some attach themselves with suckers, as in the Lernaeopodidae, others with claw-like appendages. The mouth parts are usually suctorial, the mandibles often piercing stylets enclosed in the upper and lower lips. *Argulus* has a poison spine which can be projected or withdrawn into a sheath. The anterior region of the gut is provided with extrinsic muscles for working the sucking pump.
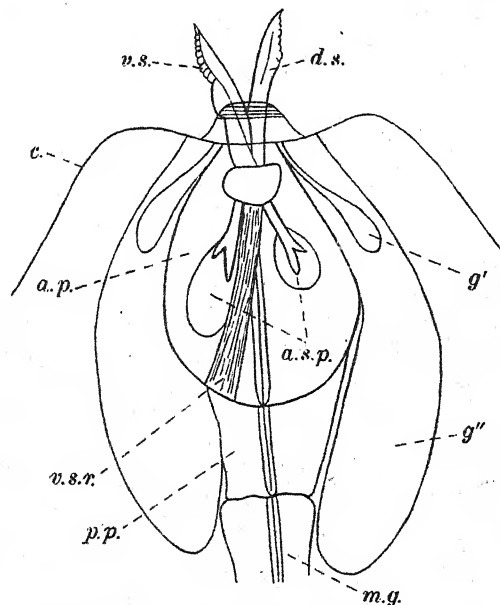
Fig. 25. *Ichthyotomus sanguinarius*, head region and pharynx. *a.p.* anterior region of pharynx; *a.s.p.* attachments of stylets in pharynx; *c.* cuticle; *d.s.* dorsal stylet; *g.' g."* glands; *m.g.* mid-gut; *p.p.* posterior region of pharynx; *v.s.* ventral stylet; *v.s.r.* ventral stylet retractor (other muscles not figured). (From Eisig (1906).)

Among the Isopods, the ectoparasitic Epicaridae have stylet-like mandibles enclosed in the upper and lower lips, which form a suctorial "oral cone."

Tardigrada. The oral cavity is surrounded by chitinous rings while there are two chitinous teeth opening into it or into the mouth tube immediately posterior to it, which pierce the walls of moss or water plants, the sap of which is sucked out by the action of the spherical, muscular pharynx.

Pycnogonida. The complicated proboscis is probably concerned with the sucking up of food, but details of the mechanism of feeding in these animals is lacking.

Insecta. Many Diptera, usually the females only, possess piercing mouth parts for sucking blood, a list of which is given by Sharp (1899, p. 457). Descriptions

C. M. YONGE

of their mouth parts and methods of feeding will be found in text-books of entomology and parasitology, as will those of the Cimicidae (bed bugs) and of the Mallophaga which, as recently confirmed by Kotlán (1923), bite into and suck the blood of birds. The larvae of the Tachinidae (Diptera) live parasitically in the bodies of other insects. Pantel (1898) has described the feeding mechanism of the larva of *Thrixion halidayanum* which consists of two chitinous rods with bifurcate roots which unite anteriorly to form a piercing beak. The pharynx is worked by a series of muscles one behind the other, the successive contractions of which, from before to behind, cause peristaltic sucking movements. An interesting case is that of the ichneumon-fly, *Habrocytus*, which, according to Lichtenstein (1921), pierces the body of its insect prey with its caudal ovidepositor, allows this to remain in the wound for about half an hour till the body fluids have coagulated around it, then withdraws the depositor and sucks into its mouth the juices of its victim through the tube so formed.

Arachnida. The parasitic mites and ticks usually possess piercing mouth parts —in the ticks, such as *Ixodes*, the chelicerae form long piercing stylets, serrated on the outer side, which open out in the wound—a proboscis and a muscular pharynx for the sucking of blood. The "salivary" glands of ticks secrete an anti-coagulin (Künssberg, 1911).

Gastropoda. According to Bruel (1904) the Nudibranchs, *Hermaea* and *Caliphylla*, which feed on Algae, such as *Codium* and *Bryopsis*, do so by sucking the soft weed into the mouth, slitting it open with the narrow radula which consists of a single row of teeth, and then sucking in the fluid protoplasm by muscular contractions and expansions of the pharynx and also of the digestive diverticula. *Doridopsis* and *Phyllidae* have each a sucking pharynx, the former having also lost the radula. The former sucks in compound Ascidians (Eliot, 1910).

(ii) *For sucking only*.

Protozoa. Plate (1889) states that the Suctorian *Ascellicola* feeds by placing its sucking tube upon its Protozoan prey, the endoplasm of which is sucked through the tube into the interior of the *Ascellicola*. This appears to be the normal method of feeding in the Suctoria.

Trematoda. These endoparasites hold on by suckers and feed with the aid of a muscular sucking pharynx on the soft tissues or juices of the host. An account of the feeding of *Fasciola hepatica* has recently been given by Müller (1923).

Nematoda. The parasitic members of this phylum suck in the semi-fluid contents of the gut of the host by means of the movements of the muscular pharynx which is triradiate in cross section.

Insecta. As examples of this type of feeding mechanism may be instanced the tubular proboscis of the Lepidoptera and the globular-ended proboscis, perforated with fine apertures, of the Muscidae (Hewitt, 1907), which are used respectively for sucking vegetable juices and any liquefiable matter. In the latter case, fluid is regurgitated from the crop when the food requires to be dissolved.

Pentastomida. These animals are parasitic in various Vertebrates, feeding

by sucking in the tissues through a small mouth which opens into a pharynx to which are attached muscles for working the sucking apparatus.

Mollusca. *Neomenia* and its allies, many of which have no radula, possess a muscular pharynx probably used for sucking in food. The solitary endoparasitic Lamellibranch, *Entovalva*, which lives in *Synapta*, probably sucks in food as it has a complete alimentary system, and also the ectoparasitic Aglossa (a group of the Taenioglossa, Gastropoda) which have neither radula nor jaws but have well-developed suctorial probosces.

(iii) *For absorption through the surface of the body.*

Under this heading come animals *without* feeding mechanisms, endoparasites which have lost feeding and alimentary systems and live in a nutrient medium in the alimentary canal or body cavity of the host, absorbing food through the general surface of the body. Examples are furnished by the parasitic Protozoa, such as the Sporozoa, all the Cestoda, the endoparasitic Gastropoda such as *Enteroxenos* and other members of the Entoconchidae, and Rhizocephalan Cirripeds such as *Sacculina*.

General remarks.

Feeding mechanisms of types (i) and (ii) have many points in common, the suction pump practically always consisting of a muscular pharynx the lumen of which is usually moon-shaped or triradiate, fluid being drawn in when the lumen is increased in size. The piercing mouth parts of type (i) usually consist of chitinous jaws or more complicated stylets often, as in many Crustacea and Insecta, guided and supported by the upper and lower lips. "Salivary" glands which produce an anticoagulin are found in many blood suckers, such as leeches, ticks, *Ichthyotomus* and perhaps *Ancylostoma*. With the exception of some of the Insects, sucking animals are usually sluggish. Feeding is usually selective and, in many of the free living and ectoparasitic forms, occasional, one good meal supporting the animal for long periods. Although a few are free living, the majority of suckers are parasitic, the transition from animals with mechanisms of type (i) to those without feeding mechanisms (type iii) corresponding closely to the change from a free living, by way of an ectoparasitic, to an endoparasitic existence. The feeding mechanisms of type (i) follow naturally upon those for external digestion. The animals here considered correspond to the suckers of Jordan and Hirsch.

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THE ASSIMILATION OF THE MOLECULAR NITROGEN OF THE AIR BY LOWER PLANTS, ESPECIALLY BY FUNGI

BY G. SENN (*Basle*).

ONE of the most remarkable results of modern science consists in the statement that the ratio of organic to inorganic compounds present on the surface of our globe always remains unaltered. At any rate, since we are able to estimate it exactly no change has taken place. This fact is the more astonishing as each organism contributes to a change of this ratio in a definite sense. Thus the quantity of carbon dioxide which is reduced each year by the assimilation of the green plants of the whole earth has been estimated by Schröder (1919) to be about 60 billion kg. In spite of this amazing quantity constantly disappearing in organisms, the amount of this gas contained in the air practically always remains unaltered because at the same time other processes such as respiration, combustion and exhalations by springs and volcanoes *produce* the same quantity of this compound. Thus there is no danger that this gas, which has a fundamental importance for the continuity of the life on our globe, should ever lack.

With respect to the ratio of the molecular nitrogen of the air to the compounds of this element, some twenty years ago the views of certain scientists, especially chemists, were not at all comforting: They saw the possibility of an irreversible decrease of the *compounds* of nitrogen, as these are constantly transformed into *molecular* nitrogen by combustion and fermentation. This process would become fatal for organisms, animals as well as plants, because neither are able to assimilate the molecular nitrogen of the air, both requiring compounds of this element, even definite compounds for each. Thus green plants need nitrates, while fungi and animals require albumen or amides, both produced by the green plants by reduction of the nitrates. Of the last-mentioned compounds large quantities occur in some countries, whilst in others they are so scanty that amongst the green plants a real struggle arises for these substances.

Apart from parasitic forms, such as pathogenic bacteria or fungi which take carbohydrates as well as nitrogen compounds from higher plants or animals, in green plants various arrangements are found enabling them to acquire the precious nitrogen compounds as well as other salts. Concerning the specialized organs of the carnivorous plants for capturing and digesting lower animals, I confine myself to mentioning the moving hairs of our *Drosera* and the pitchers of the tropical *Nepenthes*. This acquirement of nitrogen and other compounds enables *Drosera* to live in peat which is very poor in salts. To the same category of arrangements for

acquiring salts belong the hollows in the stems and roots of the so-called ant plants. As Miehe (1911, p. 356) proved for *Myrmecodia*, the inner surface of the walls surrounding those parts of the cavities in which ants deposit their faeces give a distinct reaction for nitrates, whilst the other walls of the hollows give none. These plants, living on the bark of tropical trees which are very poor in salts, would probably not be able to grow there without this interior manuring by the salts and especially the nitrogen compounds contained in the excrements of these insects.

This mode of acquiring salts from the body of animals consists in a simple *transformation* of the nitrogen compounds and preserves them for the use of the green plants. But it does not *raise* the total amount of fixed nitrogen present on the surface of the earth, any more than does the nitrification of ammonia which is performed by some bacteria (*Nitrosomonas* and *Nitrobacter*) of the soil. At any rate all those processes are not able to counterbalance the loss of nitrogen compounds produced by combustion and fermentation. Their gradual decrease would end by the general death by starvation of the green plants, and consequently of animals and men.

Long ago physicists and chemists proved that all kinds of electric discharges taking place in the atmosphere, thunderstorms, etc., oxidize a certain amount of the molecular nitrogen of the air to nitric acid. This is brought down into the soil by rain and really increases the amount of nitrogen compounds on the surface of our globe. The quantity of nitric acid produced in this way has been estimated at 5 kg. per hectare per annum (Henry, 1908, p. 209). Whether this amount suffices to counterbalance the simultaneous loss of nitrogen compounds we do not know, as we are unable to estimate the amount of the loss. At any rate the production of nitrogen compounds by electric discharges cannot counterbalance the quantity of nitrates required by the green plants growing on the same area and in the same time. These require a quantity of nitrates about 10–15 times as great.

As long as the plants and their organs remain and decay in their natural place the soil will not be impoverished of nitrogen compounds. But as soon as man takes them away by harvesting and burns or transforms them in other ways into molecular nitrogen, the danger arises of too great a loss of nitrogen compounds. Though human technique works against this loss by producing nitrogen compounds, this production cannot be taken into consideration as it is too small and too localised. In order to have a constant and infallible purveyor Nature has provided certain organisms with the faculty of assimilating the molecular nitrogen of the air.

The first naturalist to prove this by an exact method was the French physiologist Jodin. As early as 1862 (p. 613) he observed a rich development of different fungi on a substratum free from nitrogen compounds. At the same time he found that the air contained in his hermetically closed culture vessels lost a certain amount of molecular nitrogen. He estimated this to be 6–7 per cent. of the oxygen absorbed in the same time by the respiration of the fungi. As his cultures were not pure, probably containing bacteria in addition to the fungi, we cannot say which of his organisms actually assimilated the nitrogen of the air. At any rate Jodin's researches are remarkable as an early application of a gas-analytical method in the study of the circulation of nitrogen.

Whilst Jodin found the assimilation of molecular nitrogen by estimating its *loss* in a closed space, another French naturalist—the great chemist M. Berthelot—in 1885 rediscovered this faculty of low organisms independently from Jodin's researches by estimating the *gain* in nitrogen compounds in a certain quantity of natural soil. As the amount of fixed nitrogen only rose during the warm season and was definitely stopped by sterilisation of the soil, he arrived at the conclusion that this process takes place with the aid of organisms. What kind of organisms were in play he was not able to say. We may imagine that some of Jodin's fungi contributed to the increase of nitrogen compounds. Whether also the bacteria, discovered only one year afterwards by the German agronomist H. Hellriegel (Bernburg), were acting in Berthelot's experiments we do not know.

Hellriegel found (1886–9) that the faculty of the *Leguminosae* to grow in a soil free of nitrogen compounds is due to the activity of bacteria living in the tissue of the root nodules of these plants. Seedlings of *Leguminosae* planted in a sterilised soil neither develop nodules on their roots nor assimilate the nitrogen of the air. In these conditions they require nitrates as do other plants. But if they are placed in a natural soil, in which the same species of *Leguminosae* have previously been cultivated, a definite kind of bacterium enters the root hairs of the seedlings and invades the roots themselves. This invasion compels the *Leguminosae* to produce root nodules by a kind of hyperplasy, in certain cells of which the bacteria are localised. Here they receive the necessary carbohydrates from the assimilating *Leguminosae* as well as salts—with the exception of nitrogen compounds. These are produced by the bacteria themselves and forwarded to the green plant. This peaceful reciprocity in their nourishment, the so-called *symbiosis*, only lasts until the seeds of the *Leguminosae* are formed. Then its need for nitrogen compounds for storage in the seeds is so large that it simply digests its guests: the symbiosis turns into cannibalism.

While it is doubtful whether this *Bacillus radicicola* in its free-living state had contributed to the increase in nitrogen compounds in the soils studied by Berthelot, it is probable that another bacterium, the free-living *Clostridium Pasteurianum*, was there in play. Its physiology was first studied by the Russian bacteriologist Winogradsky. He published his results in 1893 and 1894, seven and eight years respectively after Berthelot's and Hellriegel's first publications. Winogradsky found that this anaerobic organism, by its fermentation performed in media containing glucose, assimilated a remarkable amount of nitrogen of the air, viz. for 20 gm. of glucose absorbed it produced 12 mg. of nitrogen compounds.

Thus there exist two groups of bacteria, which are able to assimilate the molecular nitrogen of the air, viz. the *free-living* forms on one hand and the *symbiotic* forms on the other.

Of both groups other representatives have been studied since these first discoveries. Amongst the *free-living* forms Beijerinck in 1901 (p. 567) discovered *Azotobacter* which in contrast with *Clostridium* is aerobic, decomposing glucose not by fermentation but by respiration.

Later on, other *symbiotic* bacteria forming root nodules were found in different Phanerogams other than the *Leguminosae* as *e.g.* in the tropical Conifer *Podocarpus*, in

Hippophaë, etc.* Far more interesting theoretically are those forms discovered by Zimmermann (1902, p. 4) and studied from the physiological standpoint by van Faber (1912 and 1914). These do not live in the roots but in the leaf nodules of certain Rubiaceae of Southern India and the Sunda Islands, viz. in four species of *Pavetta* (*P. indica*, *Zimmermanniana*, etc.) and in *Psychotria bacteriophila* Val. In the leaves of these plants a great number of dark green nodules are present projecting somewhat beyond the level of the leaf. From the interior of these nodules van Faber succeeded in isolating the bacteria and in cultivating them in artificial media (1912, p. 341). The analysis of his cultures proved a strong assimilation of molecular nitrogen. This explains the old experience of Indian labourers and gardeners, that the leaves of *Pavetta* are an excellent manure. In contrast with the root bacteria of *Leguminosae* those of *Pavetta* do not infect the phanerogamic plant from the soil, but are already contained in the seed between its coat and the seedling. From there they enter the interior of the leaf through the stomata and cause the formation of nodules. Later on van Faber (1914, p. 260) succeeded in cultivating bacteria-free seedlings of *Pavetta* and in infecting them with bacteria previously isolated from other individuals of *Pavetta*.

Table I. *Assimilation of the molecular nitrogen of the air.*

I	II	III	IV	V	VI	VII	VIII
		Days of culture	Glucose		Nitrogen fixation per gm. glucose		
Nr.	Organism		supplied gm.	used gm.	mg.	mg.	Investigator
1.	*Clostridium Pasteurianum*	20	40	40	53·6	1·34	Winogradsky, 1902, p. 53
2.	*Azotobacter chroococcum*	35	12	12	127·9	10·66	Gerlach und Vogel, 1902, p. 819, Kolben, No. 9
3.	*Aspergillus niger*	28	7	1·1	1·9	1·71	Ternetz, 1907, p. 388
4.	*Penicillium glaucum*	28	7	0·7	2·8	3·8	Ternetz, 1907, p. 388
5.	*Alternaria tenuis*	42	5	5·0	4·4	8·76	Froehlich, 1908, p. 295
6.	*Cladosporium herbarum*	39	5	0·48	2·9	5·95	Froehlich, 1908, p. 295
7.	*Hormodendron cladosporioides*	44	4	4·0	5·0	1·25	Stahel, 1911, p. 600·
8.	*Botrytis cinerea*	53	4	4·0	0·45	0·12	Stahel, 1911, p. 603
9.	*Phoma radicis Oxycocci*	28	7	0·85	15·3	18·08	Ternetz, 1907, p. 388
10.	*Phoma radicis Ericae*	28	7	1·1	2·3	2·17	Ternetz, 1907, p. 388
11.	*Orcheomyces Neottiae*	100	0·25	0·03	0·38	13·3	Wolff, 1926, p. 17, No. 108
12.	*Orcheomyces conopseae*	100	0·25	0·10	0·97	9·76	Wolff, p. 26

Whilst several workers (Frank, 1889, Schloesing et Laurent, 1891, etc.) have asserted that besides bacteria some green and blue-green algae were able to assimilate molecular nitrogen, the exact experiments of Schramm (1914, pp. 178–

* Some authors regard the symbionts of these plants as belonging to *Actinomyces*.

181) and others have proved that this is not the case, all of these algae having the faculty of utilising *compounds* of nitrogen alone.

On the other hand, Jodin's results, obtained (1862) with impure cultures of fungi, were confirmed later on with *pure* cultures by various workers. Thus Berthelot (1893) proved that *Alternaria tenuis* and *Aspergillus niger* assimilate molecular nitrogen, and Frank (1893, p. 146) found the same for *Hormodendron cladosporioides*. In 1895 Puriewitsch (p. 344) confirmed the nitrogen fixation in *Aspergillus niger* and found it also in *Penicillium glaucum* but only when he had added a small quantity of nitrogen compound to their culture solution. Saida (1901) examined a larger number of fungi with respect to their assimilation of molecular nitrogen and obtained positive results in three other species, viz. in *Phoma betae*, *Mucor stolonifer* and *Endococcus purpurascens*, the first of which was distinguished by an especially high power of nitrogen fixation.

Whilst these authors confined themselves to proving the nitrogen assimilation by their organisms in the usual conditions of culture, H. Froehlich (Basle, 1908) studied nitrogen assimilation in relation to different sources of carbohydrates offered to the fungi. He isolated his material from dry stems of different shrubs, on which the fungi had at their disposal a quantity of carbohydrates, especially of cellulose, but practically no compounds of nitrogen. Therefore it was to be expected that these fungi would assimilate the nitrogen of the air. For the study of this question he kept his cultures in a still atmosphere from which all nitrogen compounds had been withdrawn. Of the four species isolated in this way, two were already known for their assimilation of molecular nitrogen, viz. *Alternaria tenuis* studied by Berthelot and *Hormodendron cladosporioides* studied by Frank. In two others, *Cladosporium herbarum* and *Macrosporium commune* Froehlich proved nitrogen assimilation for the first time. Cultivating also the two moulds *Aspergillus niger* and *Penicillium glaucum* he was able to compare the behaviour of all these fungi. He found that the four species isolated from stems not only grew much better than the two moulds in media free of nitrogen compounds, but in their cultures they also produced a much greater increase of nitrogen compounds than did *Aspergillus* and *Penicillium*. He estimated this by the Kjeldahl method, and did this separately for the mycelium and for the culture solution. The latter always contained much more nitrogen compounds than the mycelium and the spores. Thus these fungi assimilate more nitrogen than they require for their own use. They evidently excrete the excess to the environment and enrich it with nitrogen compounds. It is clear that this behaviour is of great importance for the organisms growing in their neighbourhood. These fungi of course do not always assimilate the same quantity of nitrogen, as a chemical factory would do. They depend largely upon environmental conditions. As it is more practical I will treat the influence of these in connection with the publication of Stahel.

Like Froehlich, G. Stahel (Basle, 1911) isolated his fungi from dead plant material, not only from stems of shrubs, but also from old stocks of trees, dry leaves and roots. In this way he succeeded in detecting four other fungi able to assimilate the molecular nitrogen of the air, viz. *Bispora molinioides*, the common *Botrytis*

cinerea, Melanomma spec. and *Epicoccum purpurascens* Ehbg. He confirmed the
results of former naturalists who had found an assimilation of molecular nitrogen
in *Penicillium glaucum* and *Aspergillus niger*. The absolute amount of nitrogen as-
similated by these six fungi is much smaller than in Froehlich's organisms, but
sufficient for exact estimation (see below and page 83).

At any rate this amount largely depends upon the type of nutrition of the fungi.
Froehlich studied especially the influence of different carbohydrates supplied to-
gether with the necessary salts—nitrogen compounds excepted. He found glucose
to be the most suitable, though cellulose also allowed a good growth. That is easy
to understand, as Froehlich found a corrosion of the cell walls in the dry stems which
were infected by these fungi. They grew better in inuline and maltose than in
cellulose, but not so well in saccharose and lactose. Pentoses as xylose and arabinose
were a very bad source of carbohydrates for the species studied by Froehlich. In
neither of these media did the fungi show fermentation; it follows that they utilise
the carbohydrates in *respiration*. Accordingly Froehlich never found an increase of
acids in the nutritive solution, which would have been due to an imperfect oxidation
of the glucose. This negative result proves that the sugar is completely oxidated by
respiration and not by fermentation. With the energy produced by this oxidation
the fungi build up as much as 9 mg. of nitrogen compounds for each gram of
utilised glucose, whilst *Clostridium Pasteurianum* by its fermentation only produces
1·3 mg. of nitrogen compounds in the same conditions (see Table I).

Stahel studied especially the influence of an initial addition of nitrogen com-
pounds to the culture media. He found that in four of his species the assimilation
of molecular nitrogen is nearly proportional to the added amount of nitrogen
compounds. Evidently these strengthen the fungus from the beginning in such
a way that it raises the amount of its assimilated molecular nitrogen proportion-
ally.

In 1916 Duggar and Davis published a critical paper on the nitrogen fixation
in fungi. These authors paid special attention to the errors which might have in-
fluenced the results of their predecessors. On the basis of their own experiences
in the estimation of nitrogen compounds they only accept the results of Saida and
of Froehlich without any reserve. They think that Stahel's results are questionable
when the quantity of assimilated nitrogen remained below 1 mg., viz. in *Aspergillus*,
Penicillium, *Botrytis*, *Melanomma*, *Epicoccum* and *Bispora*. They believe that the
quantities of nitrogen compounds found in the cultures of these species are too
small, falling within the experimental error. The same view has been put forward
by Czapek (1920, **2**, 192) in his *Biochemie*.

In face of these criticisms I venture to remark that at least those experiments
cannot be considered to be questionable or erroneous in which the final nitrogen
contents of the cultures were markedly higher than those of simultaneous blank
experiments. As this was the case in the work of Froehlich and Stahel, as well as
in that of Ternetz (see below), I consider all of these results to be conclusive.

At any rate in all experiments with aerobic nitrogen-fixing organisms attention
must be paid to a constant aeration of the cultures, in order to supply them with

the necessary quantity of oxygen as well as of nitrogen. In these conditions the amounts of fixed nitrogen will be higher than in non-aerated cultures.

Just as in the case of bacteria there are symbiotic forms also in the nitrogen-assimilating fungi. The first were discovered by Miss Ch. Ternetz (Basle). Studying different Phanerogams of the peat she isolated from them some fungi which were able to grow without any nitrogen compounds (1904 and 1907). After having sterilised the surface of the roots of several Ericales, viz. *Erica carnea* and *Tetralix*, *Vaccinium Vitis-idaea*, *V. uliginosum*, *Oxycoccus palustris* and *Andromeda polifolia*, she obtained a development of fungi by culture of these root fragments in a moist room. All of these fungi belong to the genus *Phoma*. For one species of it, viz. *Phoma betae*, Saida (1901) had already proved the faculty of assimilating nitrogen from the air (see p. 81). Yet the species isolated by Ternetz from Ericales not only showed specific differences from *Phoma betae*, but in addition each species of Ericales was found to have its own species of *Phoma*. In *Vaccinium Vitis-idaea* Ternetz even found two different forms of *Phoma* isolated from material originating from Basle and from Freiburg (Switzerland) respectively.

As these fungi had been grown from externally sterilised roots, it was very probable that they were the fungi which form the well-known endotrophic mycorrhiza of the Ericales. In spite of this great probability Ternetz was aware of the fact that she had not directly proved the identity of her fungi with the mycorrhiza of the Ericales. Not having succeeded in growing seedlings without mycorrhiza she could not infect a fungus-free plant with its isolated fungus. It was Miss M. C. Rayner (London, 1915, p. 105) who first obtained mycorrhiza-free seedlings of *Calluna vulgaris* by opening the ripe but still closed capsules and sterilising the seeds externally from the fungus growing all around them. In this way she got fungus-free seedlings which grew, though to a height of 4 mm. only. They did not develop roots, although light and salts were at their disposal in optimal proportions. But after having been infected with the fungus previously isolated from *Calluna* they developed normally and formed their roots having now acquired their mycorrhiza from outside. Thus Rayner succeeded in proving that the fungi isolated from the roots of Ericales, which are able to assimilate the molecular nitrogen of the air, are really the mycorrhiza of these Phanerogams.

The physiology of this mycorrhiza has been studied by Ternetz with pure cultures in nutritive solutions prepared from salts containing no nitrogen, as she afterwards proved by special analysis. Like Saida she aerated the culture liquids by a slow but constant current of air liberated beforehand from nitrogen compounds such as ammonia, etc., by solutions of sulphuric acid and caustic potash. As carbohydrates she employed saccharose, mannose and glucose. As the latter produced by far the best results it was afterwards given exclusively. This predilection of the fungus is easy to understand as it is glucose which is normally passed to it by its phanerogamic host.

The fact that Miss Ternetz obtained Pycnidia-formation only if she had added nitrogen compounds to the nutritive solution is of great theoretical interest. If these compounds were not passed on, the fungi only became fertile provided they

had at their disposal an excess of oxygen. This of course, by increasing the respiration, procures for the fungus a higher amount of energy, which it applies in reducing a larger quantity of molecular nitrogen. Evidently it is only after having acquired a certain amount of nitrogen compounds—either from the nutritive solution or fixed from the air—that the fungus is able to produce its reproductive organs. At the same time these experiments prove that the assimilation of molecular nitrogen performed in the interior of the fungus requires a high amount of energy.

In the same direction these experiments give another important result. Miss Ternetz found that the higher the amount of assimilated nitrogen, the lower was the dry weight of the assimilating mycelium. Thus the fungus is not able to expend a large quantity of carbohydrates for the development of its vegetative organs if the conditions are favourable for high assimilation of nitrogen. The fungus evidently uses all the carbohydrates in its respiration, acquiring thus the energy necessary for an intensive assimilation of the free nitrogen. The excess of these nitrogen compounds is given off into the nutritive solution.

The absolute amount of these compounds is low in comparison with that produced by *Clostridium* or *Azotobacter*. But if the nitrogen compounds are reckoned on the basis of glucose used the mycorrhiza of the Ericales produces as much as 18 mg. of nitrogen compounds, whilst *Clostridium* produces not even the tenth part of this, viz. 1·34 mg. This means that the mycorrhiza of the Ericales works in a much more economical way than the anaerobic *Clostridium*. In this respect the mycorrhiza resembles *Azotobacter agilis* studied by Kostytschew and Ryskaltchouk (1925), which, like the Ericales mycorrhiza, is an aerobic organism, producing 20 mg. of nitrogen compounds for 1 gm. of glucose utilised. The superiority of the aerobic bacteria and fungi in the assimilation of free nitrogen over the anaerobic forms seems to consist in the higher amount of energy liberated from the same quantity of glucose by respiration as compared with fermentation.

The correctness of this view is also proved by the fact that the Ericales mycorrhiza when cultivated in a pure atmosphere of nitrogen which excludes all respiration only attained about one-third of the dry weight of aerobic cultures and practically did not assimilate any nitrogen at all. In spite of this anaerobic regime the mycorrhiza showed neither fermentation nor any rise of the acidity in the nutritive solutions.

All of these results prove that the mycorrhiza fungi of the Ericales are strictly aerobic and that it is only in this condition that they assimilate molecular nitrogen of the air. This assimilation allows the Ericales to live in soils such as peat and sand which are practically free of nitrogen compounds.

A second group of symbiotic fungi assimilating the nitrogen of the air has been discovered by my student Dr. H. Wolff (1925 and 1926) in the mycorrhiza of the brown *Neottia nidus avis* of our woods and in that of some green orchids of our meadows, viz. *Orchis maculatus*, *Gymnadenia conopsea*, *Helleborine (Epipactis) palustris* and *H. latifolia*. These fungi belong to the genus *Orcheomyces*, which was first isolated by Bernard (1904, p. 410) and afterwards by Burgeff (1909, p. 27) from the air roots of tropical epiphytic orchids. Wolff isolated this mycorrhiza by the same

method as Ternetz used for those of the Ericales, viz. by sterilising parts of the roots externally and putting them into sterilised water. Like the *Phomas* of the Ericales, the *Orcheomyces* developed best in *liquid* media. This is probably due to the fact that in their natural habitat in the interior of the cell they are also surrounded by liquids, viz. the protoplasm and the cell sap. *Orcheomyces* requires an uninterrupted aeration, just like the *Phomas* of the Ericales. The air, before entering the culture bottles, was freed from all compounds of nitrogen such as ammonia, etc., by caustic potash and sulphuric acid. In these conditions the different species of *Orcheomyces* studied by Wolff develop well without any compounds of nitrogen, as they are able to form them, assimilating molecular nitrogen of the air. Though the absolute quantities of assimilated nitrogen are very low, varying between 0·35 to 0·87 mg. in 100 c.c. culture solution, these values have a high degree of accuracy. As the amount of the ammonia compounds contained in the distillate of the Kjeldahl method was too small for an exact titration Prof. Kreis (Basle), who kindly performed the analysis, "nesslerised" the distillate by the methods of water analysis and estimated the ammonia content by the colorimetric method. Thus his results are exact to the tenth part of a milligram or, corresponding to the concentration of the distillate, even to the hundredth part. Therefore in the quantities published by Wolff the tenth parts of the milligrams are outside the error of estimation although the hundredths may be questionable. At any rate the fact that by this exact method no trace of nitrogen was found in the blank experiments gives good evidence that the positive results obtained with the cultures are beyond any doubt.

Like other saprophytes, Wolff's *Orcheomyces* grows in solutions of glucose containing the necessary salts (with the exception of nitrogen compounds). But as *Neottia* shows a very low assimilation of carbon dioxide (Henrici und Senn, 1925) not even sufficient to counterbalance its production of this substance by respiration, it seems to be probable that it still uses another supply of carbohydrates present in the soil. In order to find this source Wolff started cultures with solutions of different substances which may occur in the soil. Of these tannin gave the best development, the highest dry weight after a certain lapse of time being produced here.

From this glucoside the fungus splits off the glucose and lives on it, but it also grows in some way on gallic acid, the second constituent of tannin. At any rate it does not only build up its mycelium with these carbohydrates but also takes the energy from them which is necessary for the assimilation of the molecular nitrogen. Though *Neottia* itself contains tannin it is improbable that it produces this compound itself, since, as we have seen, it exhibits a very weak assimilation of carbon dioxide. As, on the other hand, Magnus (1900, p. 209) has found only a small number of hyphae connecting the mycorrhiza of *Neottia* with the surrounding soil, we have to suppose that it is *Neottia* which absorbs the tannin from the soil and forwards it to its mycorrhiza. In exchange for that *Neottia* probably obtains the nitrogen compounds, together with a relatively large quantity of carbohydrates from its fungus, because in cultures with tannin, which were not too old, Wolff

found a considerable amount of glucose which must have been produced by the fungus splitting it from the tannin.

In contrast with the glucose the nitrogen compounds produced by *Orcheomyces* are not excreted into the nutritive solution, as is the case in the mycorrhiza of the Ericales; on the contrary they are exclusively stored in the mycelium. The absolute amount of assimilated nitrogen is lower in *Orcheomyces* than in the *Phomas* of the Ericales (see Table I). But if we compare the quantities *stored* in the interior of *Phoma*, with that *produced* and completely stored in *Orcheomyces*, we find that they are of the same order. Thus Ternetz (1907, p. 385) found, in *Phoma radicis Andromedae*, 0·982 mg. nitrogen compounds, and Wolff, in *Orcheomyces conopseae*, 0·97 mg. nitrogen compounds.

The nearly complete accordance of these two quantities leads us to the conclusion that *Orcheomyces* assimilates only as much nitrogen as it wants for the production of its organs, whilst the *Phomas* of the Ericales produce an excess and excrete it to their surroundings, viz. to the living cells of the Ericales. Though in *Orcheomyces* all nitrogen compounds are stored in the mycelium, finally they too pass to the orchid which is able to digest the mycorrhiza contained in a certain layer of its tissues (Magnus, 1900, p. 256). Thus it treats its mycorrhiza just as the *Leguminosae* do their bacteria. In this way the nitrogen compounds, like the carbohydrates produced by the mycorrhiza, are used by the orchid.

The comparison of the absolute amount of the nitrogen compounds produced by Wolff's *Orcheomyces* with those produced by bacteria and the *Phomas* of the Ericales, shows that in *Orcheomyces* it is very small (see Table I), even smaller than in the common moulds *Aspergillus* and *Penicillium*. But the calculation of the ratio between the nitrogen compounds produced and the glucose absorbed (of which Chinese tannin contains 12·5 per cent.) shows that *Orcheomyces* produces relatively more nitrogen compounds than the bacteria and the moulds (see Table I, col. VII). Only the best *Phomas* of the Ericales and *Azotobacter agilis* studied by Kostytschew work in a more economical manner than *Orcheomyces*.

Thus the orchids studied by Wolff, in addition to some carbohydrates, obtain the necessary nitrogen compounds from their mycorrhiza. By the automatic production of these very important substances they are enabled to live in poor soils such as sand and peat.

Concerning this fact it is very curious that in the mycorrhizas isolated by Burgeff from the air roots of tropical epiphytic orchids, up till now *no* nitrogen fixation could be observed. If anywhere it was in *these* epiphytes, growing on the infertile bark of trees, that a nitrogen-fixing mycorrhiza would have been expected, because it would be of great advantage for its host, the roots of which are in contact with the nitrogen of the air. At any rate the presence of a nitrogen-assimilating mycorrhiza would allow us to understand why these orchids have been able to leave the soil and to become epiphytes. It is for these reasons that I am inclined to think that in suitable conditions, especially in liquid and well aerated media, the assimilation of molecular nitrogen will be demonstrated also for the mycorrhiza of the epiphytic orchids as it has been for those living in the soil.

It is seen then that a number of lower organisms exist—so far as we know exclusively bacteria and fungi—which are able to assimilate molecular nitrogen from the air and to form compounds of this element. They acquire the energy necessary for this process by respiring or fermenting different carbohydrates. With regard to the way in which they obtain the latter they may be arranged in two different biological groups, both of them containing bacteria as well as fungi.

The *first* group is represented by *free-living* saprophytic forms nourishing themselves with dead organic substances. Amongst these a great many are aerobic, obtaining their energy by respiration of oxygen (*Azotobacter, Alternaria, Hormodendron, Penicillium*, etc.), whilst others are anaerobic, acquiring their energy by fermentation of organic substances (*Clostridium*). Those substances are very different in the different species, their composition depending upon the materials which are available in their habitats. Thus Froehlich (1908) for his free-living fungi found cellulose and glucose to be the best supply of carbohydrates, whilst pentoses and plurivalent alcohols are not utilised. There is some evidence that the other organisms of this group also nourish themselves in the same manner. The fact, though, that they all develop well on glucose proves nothing, for all complex compounds are decomposed into glucose before they are absorbed.

The *second* group is represented by the symbiotic forms, which obtain carbohydrates from living plants. In the majority of cases the latter seem to deliver the carbohydrates to their nitrogen-assimilating bacteria and fungi in the form of glucose, this compound playing the chief rôle in the circulation of carbohydrates in the assimilating Phanerogams. For the mycorrhiza of the semi-saprophytic *Neottia* tannin is a better source of carbohydrates than glucose; it even seems that the tannin contained in *Neottia* is not produced by this orchid but only absorbed from the soil and delivered afterwards to the mycorrhiza. This form is also able to live on pentoses such as arabinose and xylose, whilst the free-living fungi studied by Froehlich cannot utilise these. Therefore a great variety of carbohydrate supplies exists, each of them depending upon the special habitats of the nitrogen-assimilating organisms.

As the fixation of the molecular nitrogen represents a process which in the chemical laboratory can only be performed by the application of high energies, such as electrical discharges, high temperatures or high pressures as well as metallic catalysts, the problem of the mechanism of the process in the living cell is a very old one. As early as 1894 Winogradsky tried to explain the nitrogen assimilation of his *Clostridium Pasteurianum*. After him the same question has been treated by several biologists, who examined the different reactions which seemed to be possible in the living cell. But instead of constructing a hypothesis of the chemical process acting in the living protoplasm, which is practically unknown, we shall get a better clue by examining the conditions, in which the nitrogen assimilation takes place in the plant cell.

Setting aside the anaerobic forms we may deduce from the results obtained by Ternetz that only well-aerated cultures are able to assimilate a high amount of

nitrogen, whilst in an atmosphere of pure nitrogen this gas is assimilated to a very small extent.

Stahel's results point in the same direction. He found the highest amount of nitrogen assimilation in cultures which, from the beginning, were provided with some nitrogen compounds in order to promote the development and to obtain strong mycelia. Correspondingly, Ternetz found a low dry weight in cultures which had assimilated much nitrogen. Evidently the fungus had spent the greater part of its carbohydrates in its high respiration to acquire the energy necessary for nitrogen fixation. All of these observations prove that this process can only be carried out by well-developed and strongly respiring organisms which are able to dispose of a relatively large amount of energy.

To this rule the root bacteria of the *Leguminosae* seem to form an exception. Christiansen-Weniger (1923, p. 63) deduces from his interesting researches that the nitrogen fixation of these bacteria must be an exothermic process which requires only a small amount of energy and even forwards some energy on to the bacteria. Though his deductions based upon the results of his experiments with temporary darkening (pp. 52-54) are not convincing (because he implicitly supposes the respiration of beans and oats to have the same intensity, which very probably is not the case), yet it is quite possible that this group of symbiotic organisms has a type of metabolism differing from *Phoma radicis* and *Orcheomyces* as well as from the free-living bacteria and fungi. That these require a high amount of energy for their nitrogen fixation is not contested by Christiansen.

This energy may be utilised in different ways. As we have seen Winogradsky (1894) was the first to attack the problem of the chemistry of nitrogen fixation. Observing a very strong development of hydrogen in his anaerobic and fermenting organism *Clostridium Pasteurianum* he supposed that this element in a nascent state is able to react in the interior of the living protoplasm directly with the molecular nitrogen and so to form ammonia. As this reduction of nitrogen requires a large amount of energy many authors do not admit the possibility of this reaction in the interior of the living cell. Thus Gautier et Drouin (1888, p. 823) and others suppose that in aërobic organisms an *oxidation* of nitrogen takes place to nitrous or nitric acid. On the other hand Stoklasa (1908, p. 626) thinks that cyanic acid is first formed, having occasionally found it in young cells of his *Azotobacter chroococcum*. Gerlach und Vogel (1902, p. 884) and others suppose *Azotobacter* to unite the molecular nitrogen directly with carbohydrates forming amino-acids. Czapek (1920, p. 206) supposes the formation of nitrous ammonia. Thus there is rich choice of possibilities, but no certain experimental foundation.

The only conclusive result allowing of some deductions is that obtained by Kostytschew et Ryskaltchouk (1925) who in the nutritive solutions of *Azotobacter agilis*, which showed a very intense nitrogen fixation (more than 20 mg. nitrogen compounds for 1 gm. absorbed glucose), found neither nitrous nor nitric acid, not even urea, but essentially ammonia compounds and some amines. They believe that the latter are secondary formations built up from ammonia. Assuming these compounds to be not solely derived from the primarily produced cyanic acid we may

suppose that in aerobic and perhaps also in anaerobic organisms (Winogradsky) the assimilation of molecular nitrogen goes to form ammonia directly, in spite of the great height of the lift from nitrogen to ammonia. This difficulty seems to be surmounted by the organisms with the help of the energy acquired by their vigorous respiration and probably at the same time by strong ferments present in the protoplasm.

This explanation suffers from the fact that it is based upon the nature of protoplasm, the "great unknown" with which we can explain all and nothing. Though I know perfectly well that I am not able to decide whether the direct combination of nitrogen with hydrogen is possible at the normal pressure and the normal temperature of the living cell, I should like to raise the discussion as to whether the high activity of living protoplasm may not be due to its microscopic or ultramicroscopic structure. Just as the considerable osmotic forces developed by the plant cell are due to its microscopic size, the activity of the living protoplasm may be due to its ultramicroscopic structure. As a colloid it has a very large internal surface on which the molecule of nitrogen, unable to react with hydrogen, may be split by the energy of the cell into its two atoms, which then are able to combine themselves with the hydrogen in a nascent state. Thus living protoplasm, thanks to its colloidal structure, would be able to perform in normal conditions, with the energy acquired by vigorous respiration or fermentation, a reaction for which the chemist has to employ high temperatures and pressures as well as metallic catalysts.

All the reactions considered hitherto from a mere theoretical point of view are of the greatest practical importance. Though each of the micro-organisms assimilates only a small quantity of molecular nitrogen, by their constant activity and the enormous number of individuals they fix a quantity of this gas, which is probably at least as great as or greater than the loss of nitrogen compounds caused by combustion or fermentation. Either the micro-organisms excrete the excess of these compounds during their life or after their death enrich the humus with them. In either case their nitrogen compounds become available to higher plants and animals and prevent a lack of these substances which would be fatal to plants as well as to animals and men.

Usually bacteria are considered to be the chief nitrogen assimilators. But as we have seen various fungi are also able to assimilate the nitrogen of the air. Their great importance for the economy of Nature can not only be appreciated in a theoretical way, but it has already been proved by practical experiments on a big scale (Henry, 1908, pp. 219 ff.). Thus the sand-dunes of south-western France (Gascony) which before had no vegetation at all were planted with *Pinus maritima* and developed during about fifty-six years a fine wood of medium density. In analysing the sandy soil it was found that the micro-organisms which developed in it owing to the presence of the dead leaves of the pines had produced in these fifty-six years 270 kg. of nitrogen in each hectare or 5 kg. each year.

A complementary experiment carried out with a pure silicic sand planted with *Pinus maritima* and *Laricio* aged nine years confirmed this result. After ten years the superficial layer of the sand containing some remains of dead leaves was transformed into a kind of felt by the brown hyphae of a fungus resembling a *Cladosporium*

(see Table I, No. 6). The gain of nitrogen amounted even to 8 kg. for one hectare.

Considering these remarkable results Henry (1908, p. 220) is right in saying that: "A wood represents the best saving-box for nitrogen compounds."

The same is true of course for the associations of plants which contain a nitrogen-fixing mycorrhiza, for example the large heaths covered by different species of the Ericales, *e.g. Calluna vulgaris, Erica carnea* and *E. tetralix, Vaccinium myrtillus*, etc. Like these the alpine roses (*Rhododendron*) in the European Alps as well as in the high mountains of Asia (Himalayas, Ceylon) enrich the soils by the action of their nitrogen-assimilating mycorrhiza. In the large brushwoods (*macchia*) of the Mediterranean countries, the strawberry tree (*Arbutus*), containing a mycorrhiza which probably also assimilates molecular nitrogen, acts in the same way. Thus these shrubs and bushes of the Ericales, generally considered to be of no use, are of great importance in maintaining the equilibrium of molecular and compound nitrogen. The same is the case for the orchids, at least if they occur in a sufficient number of individuals, as is sometimes the case in our meadows, swamps or in-fertile limestone districts.

This short review proves that nearly every country has not only its nitrogen-assimilating bacteria but also its nitrogen-fixing fungi. As far as we know the fungi are only absent in the sea and lakes, for which Keutner (1905, p. 47) and others have proved the presence of nitrogen-fixing bacteria. In spite of this restriction regarding the hydrosphere, a certain group of fungi is, like the bacteria, of great importance in the assimilation of molecular nitrogen. By the constant production of nitrogen compounds these organisms make nitrogen available to the vegetation of higher plants and indirectly to animals and men, which have no direct access to the nitrogen of the air.

Just as green plants by assimilating carbon dioxide maintain the equilibrium of this substance and regulate its circulation, so fungi as well as bacteria by assimilating the molecular nitrogen of the air maintain the quantity of nitrogen compounds constant on the surface of the earth. Electric discharges in the atmosphere assist in this process of nitrogen regulation. Thus fungi, by the part they play in the circulation of nitrogen, aid in realising one of the chief conditions for the continuity of life.

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L' ANAFILASSI
DA UN PUNTO DI VISTA BIOLOGICO
DI ENRICO SERENI
Capo del Reparto di Fisiologia della Stazione Zoologica di Napoli.

INDICE.

INTRODUZIONE.

RICORRE quest' anno il venticinquennio dalla pubblicazione della memoria nella quale Richet e Portier (Richet e Portier, 1902) davano la prima descrizione metodica del fenomeno al quale il Richet assegnava fino da allora il nome, del resto poco felice, seppure tanto fortunato, di anafilassi. Le ricerche bibliografiche successive hanno dimostrato che altri autori, già prima di essi—e basta fra questi ricordare il Magendie—avevano descritto dei fenomeni analoghi: ma nessuno ne aveva intuito la importanza o tentato una spiegazione generale: sicché ai due autori francesi spetta l' onore incontestabile di avere aperto un campo di ricerche che é stato fra i più battuti e fecondi del quarto di secolo che ha seguito il loro esperimento fondamentale.

Col moltiplicarsi delle ricerche, il quadro si é venuto sempre più complicando e, si può dire, oscurando: sicché oggi si discute vivamente sulla stessa definizione di

anafilassi e sui fenomeni che in essa possono esser compresi: dall'una parte richiamandosi alle prime definizioni date, necessariamente larghe ed indistinte nella loro lettera, per comprendere nell'anafilassi fenomeni che a prima vista sembrano da questa lontani per natura e diversi per meccanismo: dall'altra invocando le restrizioni, più inespresse e sottintese che esplicite, che a quelle definizioni furono quasi sempre imposte, per escludere dal novero delle manifestazioni anafilattiche sindromi e fenomeni che ad esse sicuramente appartengono.

In queste condizioni, e in una materia tanto complessa, il compito del critico si presenta assai scabroso, ma non poco interessante, né inutile: che é solo da una critica, serena ma serrata, dei dati dei molti autori, che é possibile giungere ad una qualsiasi concezione unitaria dei fenomeni in esame; anche se l'esperienza ci dimostra che tutti i tentativi passati e probabilmente, e per molti anni, anche quelli avvenire, sono serviti e serviranno soltanto ad indicare nuove vie di ricerca, e con questo a complicare il problema, e non già a risolverlo.

DELIMITAZIONE DEL CONCETTO DI ANAFILASSI

Prima di procedere ad una più accurata considerazione dei punti che ci interessano più da vicino, occorre quindi delimitare per quanto é possibile il campo da esaminare.

Gli Aa., come si é già detto, sono in proposito tutt'altro che concordi; e ciò deriva per un lato dal fatto che allo studio dell'anafilassi hanno partecipato, quasi in egual misura, seppure in tempi diversi, studiosi di varia provenienza ed educazione (fisiologi, patologi, batteriologi, immunologi, clinici) che la differente formazione mentale portava a dare particolare preminenza all'uno piuttosto che all'altro gruppo di fenomeni. D'altra parte sembra in effetto che anche in questo, come in quasi tutti i campi della biologia, i limiti non siano così netti come sarebbe desiderabile: sicché il tracciare il confine un poco più lontano o più vicino racchiude sempre in sé qualcosa di arbitrario. Infine in molti punti ed in molte questioni le ricerche più recenti hanno condotto a profonde modificazioni delle concezioni precedenti: e resta a decidere, caso per caso, quando queste modificazioni siano tali da render necessario un ampliamento, o una restrizione, dei limiti dei fenomeni da considerare nel capitolo dell'anafilassi: e quando invece esse portino ad un tale mutamento della precedente posizione da indurre ad una esclusione dal quadro dei fenomeni anafilattici. Anche quì, é difficile evitare di cadere almeno nell'apparenza dell'arbitrio; ché, secondo che si darà considerazione preminente ad uno o ad un altro dei momenti dell'anafilassi, si sarà condotti ad escludere una classe od un'altra di fenomeni: ma quello di cui appunto si discute é quale sia il quid caratteristico dell'anafilassi, che permetta senz'altro di riconoscerla con la sua presenza, e di escluderla, nella sua assenza.

Ad aumentare l'incertezza e la confusione su questo punto ha contribuito non poco un caso disgraziato: quello cioé che le prime ricerche del Richet sul cane furono condotte con una sostanza di per sé tossica: e del pari quelle di Theobald Smith (1906) sulla cavia. Vero é che, fin dalla loro prima comunicazione, Richet e Portier misero bene in luce che i fenomeni che si ottenevano alla seconda iniezione

erano diversi da quelli provocati dalla prima, ed eguali per le diverse sostanze impiegate: ma ciò non toglie che il fatto indicato abbia influenzato, più o meno inconsciamente, oltre molti degli autori successivi, gli stessi primi scopritori; e che a questo fatto sia dovuta probabilmente l' origine di due termini improprii, quali anafilassi ed ipersensibilità. Di questi il primo implica il concetto di protezione—attesa, non raggiunta: é bene qui ricordare che lo stesso Richet aveva per la prima volta osservato il fenomeno (Richet e Héricourt, 1898), senza, peraltro, comprenderne l' importanza, durante alcuni tentativi rivolti ad immunizzare il cane rispetto al siero di anguilla—ed introduce perciò un concetto teleologico per lo meno inutile. D' altra parte essa porta ad una contrapposizione ai fenomeni dell' immunità la quale, come si vedrà in seguito, é priva di ogni seria base; ed urta contro quei criteri patogenetici ai quali dobbiamo sempre cercare di ispirare ogni classificazione di fenomeni biologici.

Quanto alla parola "ipersensibilità", anche essa non appare troppo esatta. A meno infatti di intenderla in un senso del tutto vago e generico, che sembra poco consono alle buone regole scientifiche, non é proprio parlare di aumentata sensibilità—che implica un' azione qualitativamente identica sebbene quantitativamente differente—a proposito di fenomeni la sintomatologia dei quali può essere del tutto diversa da quella provocata dalla prima iniezione: mentre d' altra parte, come ha messo bene in luce il Doerr (Doerr e Bleyer, 1926; Doerr, 1926, 2), durante lo choc l' animale non reagisce allo stimolo iniziale (l' antigene), ma ad un nuovo stimolo (la reazione antigene-anticorpo) per il quale l' organismo é sempre ugualmente sensibile. É per questo che l' esperimento dell' anafilassi passiva ha una importanza straordinaria nello studio dell' anafilassi: in quanto esso ci consente ad ogni momento la dimostrazione dello svolgersi di questa reazione in condizioni di semplicità ideali, nelle quali l' organismo anafilattizzato passivamente funziona soltanto da indicatore di una reazione. Che, in questo senso, la sensibilità non si modifichi, é dimostrato, p.e., dalle ricerche nelle quali si é praticata la anafilattizzazione per 2 o più antigeni; e nelle quali si é potuto dimostrare che é possibile provocare, con i diversi antigeni, diversi chocs successivi (Massini, 1918; Brack, 1921); e da quelle nelle quali, subito dopo lo choc, l' organismo vien di nuovo sensibilizzato passivamente con risultato positivo. Non si tratta quindi di sensibilità aumentata; ma, da un lato, di sensibilità costante (alla reazione antigene-anticorpo); dall' altra dell' insorgenza, provocata dalla prima iniezione dell' antigene, di una nuova proprietà dell' organismo: e cioé—se non si vuole pregiudicarne il meccanismo—della proprietà di dar luogo alla detta reazione.

LO CHOC.

Quello che siamo venuti dicendo implica alcune conseguenze, che ci consentono una prima delimitazione del campo del nostro esame.

(*A*) Lo choc non é il momento essenziale del processo anafilattico, ma soltanto la dimostrazione della sua esistenza. Lo stato anafilattico sussiste prima e può sussistere sempre, all' infuori della produzione di uno choc: e, come si vedrà in seguito, anche a prescindere dai brutali fenomeni dello choc, l' organismo anafilat-

tizzato si presenta profondamente modificato, e non soltanto nei riguardi dell' antigene.

(*B*) Se lo choc non é il momento essenziale del processo anafilattico, ma soltanto un suo epilogo non necessario, ne segue che il suo meccanismo patogenetico ultimo non ha alcuna importanza per decidere dell' esistenza di una sindrome anafilattica. Il problema che ci si offre in questo caso é quello, interessantissimo, dell' azione sull' organismo di un agente nocivo, del quale per il momento non approfondiremo la natura: ma non ha nulla a vedere col problema dell' anafilassi, che é invece quello del meccanismo di produzione di questo agente nocivo.

(*C*) Da quel che si é affermato in (*B*), segue che il meccanismo dello choc può essere diversissimo in diversi animali, senza che questo debba far ritenere che si tratti di fenomeni diversi nella loro genesi primitiva. Le condizioni di eccitabilità dei varii tessuti ed organi sono sicuramente differenti nei diversi animali: onde é naturale che questi possano reagire differentemente allo stesso stimolo (come ci é noto da innumerevoli esempi). Né, d' altra parte, é necessario ammettere che lo stimolo sia identico nei diversi animali: ché identità nel meccanismo di formazione non significa necessariamente identità nel risultato finale, perché sono sicuramente differenti da animale ad animale le condizioni iniziali.

(*D*) Da quel che si é detto in (*B*) consegue ancora che una somiglianza, od anche, se vi fosse, identità dei fenomeni di choc, non può mai autorizzare a concludere sulla natura anafilattica o meno di una sindrome. Le possibilità, i modi di reazione di un organismo ad un qualsiasi agente nocivo sono limitati. Noi sappiamo perfettamente che lo stesso fenomeno, p.e., la contrazione di un muscolo, può essere provocato in molte diverse maniere: é pur tuttavia a nessuno parrebbe per questo lecito di identificare i diversi stimoli che l' hanno provocato. Questo é invece il criterio seguito nel campo dell' anafilassi da molti ricercatori, specialmente clinici; i quali, da una più o meno profonda somiglianza di quadri sintomatici, conclusero spesso precipitosamente per la natura anafilattica dei più diversi fenomeni. Conclusioni di questa sorta appaiono e sono del tutto illegittime: e se pure, col diminuire della "moda" dell' anafilassi, e con la sempre rinnovata constatazione della loro fallacità, esse si fanno sempre meno frequenti, pure é bene insistere sullo scarso valore dimostrativo delle ricerche condotte in questa direzione. In particolare é da indicare qui il valore scarso o negativo, dal punto di vista dell' anafilassi, dei diversi fenomeni di choc colloidoclasico, messi in luce specialmente dagli autori francesi. Che nella fenomenologia dello choc le modificazioni dei sistemi colloidali dell' organismo possano avere una importanza notevole, nessuno vuol negare: e non é questo il luogo del dibattito; ma occorre mettere bene in luce il danno che la sovravalutazione di queste indagini, che consideravano esclusivamente un sintomo, sia pure conclusivo, ha portato allo studio di quello che é il problema fondamentale dell' anafilassi, cioé il processo di sensibilizzazione.

Anche in questo caso una indagine storica, pur se sommaria, mostra quale sia stata la via che ha condotto a questo indirizzo pericoloso e sterile. Fu attraverso le indagini rivolte a confermare o a controllare la scoperta dell' anafilatossina del Friedberger che furono fatte le prime constatazioni della provocabilità di sindromi

choc-simili in seguito a modificazioni fisico-chimiche del siero. Dopo di allora, si é troppo spesso dimenticato che quello del Friedberger, e a maggior ragione gli altri che ne derivavano, non erano e non potevano essere se non tentativi di spiegazione del meccanismo dello choc, o meglio della produzione dell' agente nocivo provocatore dello choc; ma che essi non risolvevano e neppure toccavano il problema fondamentale dell' anafilassi, e cioé il processo della anafilattizzazione.

(*E*) Se l' identità della sindrome finale non può essere un criterio sufficiente per affermare la natura anafilattica di un fenomeno, se quindi la sua esistenza non ha un valore positivo, la sua assenza, e l' impossibilità di provocarla anche nelle più adatte condizioni sperimentali, depongono contro la natura anafilattica del fenomeno osservato.

É infatti caratteristico della sindrome anafilattica di presentarsi con modalità differenti nelle diverse specie, ma identiche negli individui della stessa specie, qualunque sia la sostanza anafilattizzante. Questa ultima affermazione va intesa con intelligenza. Non si vuole con essa affermare la unicità e la immutabilità della sindrome anafilattica in tutti gli individui di una stessa specie ed in qualunque condizione; a tutti é infatti noto che i caratteri dello choc variano nel medesimo animale, in relazione a molti fattori, p.e., la via della seconda introduzione. L' affermazione deve perciò esser compresa nel senso che, a parità di altre condizioni, la sindrome deve essere identica anche se la sostanza anafilattizzante é diversa.

(*F*) Come corollario dell' affermazione fatta in (*E*), é bene ricordare che la sintomatologia provocata dalla seconda introduzione della sostanza anafilattizzante é generalmente diversa da quella provocata dalla prima introduzione. Questo si comprende facilmente data la identità dei sintomi provocati alla seconda introduzione da tutte le sostanze, qualunque sia la loro azione primitiva. D' altra parte si possono avere sostanze anafilattizzanti che già ad una prima introduzione danno una sindrome choc-simile: e questo si comprende, dato il numero relativamente limitato di modalità di reazione che l' organismo può presentare. In questo caso, naturalmente, il fenomeno si rivelerà attraverso la notevolissima diminuzione delle dosi necessarie per provocare la sindrome.

LA SENSIBILIZZAZIONE ED IL PERIODO DI INCUBAZIONE.

Lo choc non può quindi essere considerato l' elemento caratteristico dell' anafilassi: ma anzi come un fenomeno conclusivo, banale quanto imponente, che specialmente nella cavia, l' animale più spesso impiegato in queste ricerche, può essere provocato con numerosissimi mezzi; sicché, invece di considerare sempre la constatazione di uno choc come prova di uno stato anafilattico, si dovrà esser ben cauti e non ritenere di origine anafilattica se non quegli chocs che occorrono in condizioni particolari.

Prima fra queste é che la provocazione dello choc per mezzo di una determinata sostanza deve esser stata preceduta, qualche tempo prima, dalla introduzione (di regola parenterale) di quella stessa sostanza. In altre parole, l' attribuzione all' anafilassi di fenomeni che si verificano alla prima introduzione di una determinata

sostanza si deve in linea generale considerare ingiustificata ed arbitraria: e rende necessaria, salvo alcuni pochi casi particolari (quello, per esempio, della cosidetta anafilassi rovesciata, nella quale si ottiene uno choc iniettando ad un animale nuovo il siero di un animale preparato contro di esso), l' ammissione di un' introduzione precedente, sfuggita al nostro controllo, della stessa sostanza.

Non basta però che sia avvenuta una precedente contaminazione dell' organismo con quella stessa sostanza: occorre che essa si sia verificata con un intervallo di tempo determinato. Lo stato anafilattico é perciò preceduto da un periodo di incubazione la durata del quale varia con la via della prima introduzione, con la dose di questa, con la specie animale, con la natura dell' antigene, etc.

Questa affermazione deve però essere intesa con discrezione, e non ha un valore assoluto; quanto più delicati sono i metodi di indagine, e tanto più breve é il periodo d' incubazione, quello cioé durante il quale una seconda introduzione dell' antigene non provoca nell' animale fenomeni differenti dalla prima: sicché si può ritenere che l' animale cominci a divenire anafilattico dal momento stesso della prima introduzione. Che così debba essere, appare evidente secondo le teorie del Friedberger, suffragate da esperimenti suoi e di altri autori, sulla formazione degli anticorpi per via riflessa (vedi però le esperienze contrarie del Cohn (1926)): mentre se si accetta la teoria corrente, sembra più logico pensare che la produzione degli anticorpi e lo stato anafilattico si inizino soltanto dopo che l' antigene introdotto é stato fissato dalle cellule produttrici degli anticorpi medesimi. Ma che la modificazione dello stato dell' organismo sia assai precoce é dimostrato da un fatto assolutamente generale: e cioé una seconda introduzione dell' antigene, che segua anche a brevissima distanza la prima, modifica, rallentandolo, il processo della sensibilizzazione; in altre parole, l' organismo reagisce a questa seconda introduzione in maniera differente che alla prima. Questo fatto permette di spiegare come l' introduzione di dosi maggiori di antigene si risolva in un prolungamento del periodo di incubazione: si può infatti pensare che le primissime aliquote di antigene fissato dalle cellule inizino senz' altro la sensibilizzazione di queste; sicché il resto dell' antigene viene ad agire su cellule già modificate, e si viene perciò a trovare in condizioni analoghe a quelle dell' esempio precedente.

Se ora la introduzione dell' ántigene é proseguita per un tempo sufficientemente lungo, la provocabilità dello choc sarà sempre più dilazionata: e l' animale apparirà immune.

Derivano da questo due conseguenze, ambedue assai importanti: in primo luogo, la necessità, perché si stabilisca l' anafilassi, che alla prima introduzione dell' antigene, in dose adeguata, succeda un periodo durante il quale non avvengono nuove introduzioni di esso.

La seconda conseguenza é che non é possibile, o estremamente difficile, tracciare un limite netto fra i fenomeni di anafilassi e quelli di immunità: e che gli uni e gli altri devono esser compresi in una più vasta classe di fenomeni, caratterizzati da una modificazione della reattività dell' organismo rispetto a stimoli specifici, che consegue a un primo trattamento con essi, e che potremo chiamare allergia. Il contrasto dei due ordini di fenomeni appare infatti evidente soltanto quando si consideri, con

procedimento assolutamente ingiustificato, esclusivamente il loro momento finale: mentre la loro fondamentale unità si mostra in chiara luce appena si proceda ad una analisi più accurata.

La modificazione della reattività specifica deve quindi essere considerata come il fenomeno fondamentale con il quale gli organismi reagiscono ad un primo trattamento con una sostanza antigene: e l' anafilassi non é che una modalità di questo fenomeno, che ci appare più strana e "curiosa" (usiamo le parole stesse del Richet, 1923, pag. 1) dell' altra solo perché essa non risponde, almeno apparentemente, ad alcun criterio teleologico preciso, quale é immediatamente riconoscibile, p.e., nell' immunità. Ciononpertanto, immunità ed anafilassi sono fenomeni assai vicini, perfettamente equivalenti, e che rappresentano il modo di reagire a stimoli specifici di un organismo già modificato da un primo intervento. Non é quindi soltanto la reazione che é modificata, ma essa lo é, in quanto la nuova applicazione dello stimolo avviene in un organismo modificato, diverso da quello sul quale il primo stimolo aveva agito. Lo stabilirsi di uno stato di immunità o di anafilassi non può quindi essere interpretato come espressione di una difesa che l' organismo fa della sua individualità biochimica contro l' azione di agenti diversi ed esterni ad esso: ma anzi come un aspetto delle azioni molteplici che questi agenti hanno esercitato, tali che l' organismo ne sarà per un tempo più o meno lungo, molto spesso per sempre, modificato. Che questa modificazione non sia limitata ai soli rapporti con l' antigene, e nei riguardi dell' apparato immunitario (se ci si concede questa espressione) é dimostrato da molti fatti, che tendono a stabilire come molti organi si trovino, per il solo fatto della sensibilizzazione, in condizioni nuove (v.p.e. Hashimoto e Pick, 1913, 1914; Kling, 1912, etc.).

L' ANTICORPO ANAFILATTICO.

Fra i diversi organi e tessuti uno ve ne é a proposito del quale le modificazioni sono state studiate con maggior cura e sono quindi note con maggiore esattezza: il sangue.

Per quel che riguarda i suoi componenti morfologici, é noto, dalle ricerche del Metalnikow (1922), che i leucociti di animali sensibilizzati sono sensibilizzati essi stessi: né, d' altro lato, vi é bisogno di ricordare la parte che i leucociti prendono ai fenomeni dell' immunità. Anche nei riguardi del plasma, le modificazioni non sono di solito meno profonde: a parte le variazioni dei diversi poteri fermentativi, e di molte altre proprietà, rivelabili già durante l' incubazione, dopo un certo periodo il plasma acquista il potere di trasmettere l' anafilassi (o l' immunità) ad altri animali, non trattati precedentemente. Questo fatto importantissimo, e che stabilisce in maniera definitiva che lo choc é un fenomeno basato sulla reazione antigene-anticorpo, é stato anche l' origine di una discussione non ancora ben chiusa e che per lunghi anni é stata causa di infinite ricerche e di dispute non meno infinite: quella cioé sulla sede umorale o cellulare della reazione anafilattica. In effetti, appare chiaro che la sede ultima di ogni azione non può essere che cellulare; sicché il problema non é tanto relativo alla sede d' azione quanto a quella di reazione fra antigene ed anti-

corpo. Ora, che in molti casi si possano avere reazioni anafilattiche in animali o in tessuti più o meno completamente privati del loro sangue e degli anticorpi che con questo circolano, é indubitato e indubitabile: ma d' altra parte appare poco probabile che la reazione fra gli anticorpi circolanti nel sangue e l' antigene possa risultare completamente innocua, quando in molti altri tessuti la stessa reazione porta a tanto gravi conseguenze. Lo stesso Doerr infatti, che é sempre stato fra i più strenui sostenitori della sede cellulare dei fenomeni dello choc, ammette recentemente (Doerr, 1926, 1 e 2) che i fenomeni umorali possano agire citotossicamente su gli endotelii e sui muscoli lisci, provocando così sintomi anafilattici. Ma questi fenomeni non sono a stretto rigore anafilattici, e corrispondono piuttosto a quelli che molti autori hanno ottenuto facendo agire, in vitro ó in vivo, su animali interi o su organi isolati, i prodotti della reazione fra antigene e anticorpo.

Gli effetti della reazione che si svolge nel sangue fra gli anticorpi anafilattici e gli antigeni relativi devono essere cercati nel sangue stesso: e sono in effetti rivelabili in diversi fenomeni, quali, per citarne solo alcuni, l' enorme diminuzione o addirittura la scomparsa della coagulabilità del sangue: le profonde modificazioni delle proprietà fisico-chimiche di questo (Segale, 1911, 1912) l' aumento del potere antitriptico (Rusznyak, 1912).

Il sangue in seguito a queste considerazioni vien tolto dalla posizione singolare nella quale esso era stato lasciato dai diversi autori, e viene considerato alla pari di tutti gli altri tessuti, come uno di quelli nei quali la reazione anafilattica può svolgersi. Le conseguenze di questa reazione si rivelano, come si é già detto, nel sangue stesso: i diversi fenomeni chimico-fisici dimostrati nel sangue al momento dello choc sono una parte di esse: mentre i fenomeni che si svolgono in altri tessuti, e che non siano riportabili a reazioni *locali* fra antigene e anticorpo, sono secondari, e come tali non direttamente connessi con la reazione anafilattica, ma epilogo e conseguenza di questa.

Considerando le cose in questo modo, e dato che non é dubitabile che anticorpi circolino nel sangue di molti animali, questo non diventa che uno dei tanti possibili organi o tessuti dello choc (dando questo nome a quegli organi o tessuti nei quali la reazione antigene-anticorpo si svolge con particolare violenza di effetti). Se é così, però, é evidente che, come avviene per molti altri tessuti, esso può, in determinati animali o in determinate condizioni, esser privo di anticorpi, incapace quindi di reazione: e la dimostrazione di anticorpi circolanti nel sangue cessa di essere un criterio generale dell' anafilassi (e dell' immunità). É già noto, p.e., che la cavia sensibilizzata resta anafilattica per lunghissimo tempo, probabilmente per tutta la vita: mentre non é possibile dimostrare anticorpi nel suo sangue se non per un tempo relativamente breve dopo la sensibilizzazione (circa 70 giorni). Nulla si oppone ad ammettere che quello che nella cavia si verifica soltanto in un determinato periodo (stato anafilattico senza anticorpi circolanti) possa in altri animali verificarsi sempre. Da un punto di vista più generale, nulla ci costringe ad ammettere che gli anticorpi formati dalle cellule per l' azione di un determinato stimolo debbano necessariamente circolare; ma si può senza difficoltà pensare che essi restino costantemente sessili: sicché la mancata constatazione della comparsa di anticorpi

nel sangue di un animale non può autorizzarci in nessun modo a concludere che quell' animale non é capace di produrre anticorpi[1].

Il metodo di constatazione degli anticorpi attraverso i loro effetti in vitro (precipitazione, agglutinazione, etc.) fra molti vantaggi, quali, p.e., la facilità di un dosaggio accurato, presenta il grave inconveniente di non permettere conclusioni definitive: e da questo punto di vista é assai superiore l' esperimento dell' anafilassi, che permette la constatazione della reazione antigene-anticorpo in vivo; e rivela perciò anche gli anticorpi rimasti sessili. In ogni modo, é bene ripeterlo ancora una volta, mentre sembra assolutamente sicuro che la reazione anafilattica sia una reazione fra antigene ed anticorpo, nulla richiede di ritenere la comparsa dell' anticorpo nel sangue come un sintomo essenziale dell' anafilassi (ed, in genere, la comparsa di anticorpi circolanti come un sintomo necessario di una reazione immunitaria).

La presenza frequente degli anticorpi nel sangue di molti animali é probabilmente collegata al fatto che essi sono principalmente, se non esclusivamente, prodotti negli organi ematopojetici; sicché l' anafilassi degli altri organi e tessuti in un certo senso é un' anafilassi passiva, ed il sangue rappresenta il veicolo che porta gli anticorpi dagli organi produttori agli organi fissatori, in corrispondenza dei quali si svolgerà più tardi la reazione. Esiste una netta distinzione ed indipendenza fra queste due specie di organi o, se si preferisce, fra queste due funzioni. La formazione degli anticorpi procede, p.e., già nei primi giorni dopo lo choc, mentre l' animale, cioé i suoi organi dello choc, sono per parecchio tempo incapaci di reagire (Otto, 1907). I nostri dati sui luoghi di produzione degli anticorpi sono però tutt' altro che definitivi; ed in ogni modo essi sono stati sempre raccolti in poche specie di mammiferi, di solito vicine fra loro. Nulla sappiamo riguardo agli altri animali: e si può perfettamente ritenere che le condizioni non siano ovunque le medesime.

ALTRI CRITERII DELL' ANAFILASSI.

Dopo aver proceduto alla esclusione di alcuni criteri, quali, p.e., lo choc, abbiamo ora riconosciuto almeno due elementi, la presenza dei quali é necessaria perché si possa parlare di anafilassi o meglio ancora di allergia, comprendendo sotto questo nome i fenomeni di anafilassi e di immunità; l' esistenza cioé di una precedente introduzione della sostanza anafilattizzante, e di un periodo di incubazione. Abbiamo visto anche come la reazione anafilattica sia essenzialmente una reazione fra antigene ed anticorpo: ma che la presenza di anticorpo nel sangue non é un elemento costante e necessario. Resta ora a vedere se si possa scoprire qualche altro carattere necessario all' affermazione dell' esistenza di uno stato anafilattico.

(a) LA SPECIFICITÀ.

Alla nostra considerazione si presenta subito un primo elemento, che siamo già andati accennando più volte, ma sul quale é ora opportuno fissare un poco l' attenzione: e cioé quello della specificità della reazione anafilattica. Fenomeni ana-

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[1] Recentemente il Doerr (1924) ha esposto delle considerazioni analoghe; ed é riuscito a scoprire una forma di anafilassi della cavia (l' anafilassi da eritrociti) nella quale la trasmissione é sempre impossibile; e nella quale perciò non si può dimostrare la presenza di anticorpi nel sangue.

filattici si ottengono infatti soltanto quando la sostanza introdotta la seconda volta é identica a quella usata per la sensibilizzazione. I casi nei quali ad una prima introduzione di una determinata sostanza succede una diminuita resistenza verso molti agenti sono casi che nulla hanno in comune con i fenomeni anafilattici, per quel che riguarda la sintomatologia, e che non sono dovuti ad una reazione fra antigene e anticorpo. Il fatto che talvolta é possibile ottenere fenomeni più o meno gravi iniettando in animali sensibilizzati sostanze vicine, ma non identiche, all' antigene, non infirma il valore di questa specificità: ché da un lato noi conosciamo da gran tempo la possibilità, in tutti i fenomeni immunitari, di queste reazioni di parentela, dall' altro le ricerche quantitative esatte permettono sempre di stabilire che l' organismo sensibilizzato reagisce più intensamente all' introduzione dell' anafilattogeno che a quella di qualsiasi altra sostanza. La specificità della reazione entra quindi a far parte dei criteri necessari alla diagnosi di anafilassi.

(b) GLI ANAFILATTOGENI.

Un altro criterio può essere cercato nella natura della sostanza anafilattizzante. Noi sappiamo che nella grande maggioranza dei casi conosciuti questa é rappresentata da una proteina; ma é pur noto che assai numerose, e sempre rinnovate, e sempre pure contraddette sono le affermazioni di aver ottenuto l' anafilassi con sostanze diverse dalle proteine e ritenute comunemente non antigeni; quali lipoidi (vedi gli ultimi risultati, parzialmente positivi, di Klopstock (1926) e Polettini (1926, 1927)), e sostanze a composizione chimica ben definita, da sole (v.p.e., per l' ursolo, Curschmann (1921) e Gerdon (1920); ed i risultati negativi del Meyer (1925)), o insieme a proteine (Meyer e Alexander (1924) con atoxyl; Landsteiner con azo-composti). In altri casi l' azione sensibilizzante deve probabilmente, secondo l' antica ipotesi del Wolff-Eisner, essere attribuita alle proteine dell' organismo stesso, più o meno profondamente modificate dall' azione della sostanza introdotta. Questo é almeno quello che sembra essersi verificato negli esperimenti del Richet con cloroformio e fosforo (Richet, 1914); ed in quelli più recenti del Henrijean et Kopaczewsky con acque ferruginose (Henrijean et Kopaczewsky, 1925); ammesso per questi ultimi che si tratti di anafilassi e non di fenomeni banali, come vuole il Peeters (1926).

In ogni modo nei mammiferi, ed in genere nei vertebrati, sembra che, se pure vi sono, i casi di anafilassi sperimentale per sostanze non proteiche rappresentino l' eccezione: é anzi essenzialmente sulle proprietà non antigene delle sostanze provocatrici che alcuni autori han fondato l' esclusione dal gruppo delle reazioni anafilattiche di molti fenomeni, quali, p.e., le idiosincrasie medicamentose dell' uomo.

Conclusioni di questo genere non sembrano però oggi del tutto autorizzate: che recentemente si é riusciti a dimostrare il potere antigene di sostanze per le quali non lo si ammetteva finora (Walzer e Grove, 1925, per il polline); e, con tecniche più adeguate, si é potuta rilevare quasi costantemente la presenza di anticorpi in molti casi nei quali essa non era fino ad oggi riuscita (Prausnitz e Küster, 1921; de Besche, 1923; Coca e Grove, 1925; Walzer e Kramer, 1925; Levine e Coca, 1926) e specialmente nel gruppo assai vasto delle affezioni asmatiche e simili dell' uomo.

Negli ultimi mesi, il Melli (1927) é riuscito a dimostrare che il siero di individui che presentano una reazione cutanea positiva, saggiato con la tecnica dell' Abderhalden, dá una reazione positiva con gli estratti di polline.

Mancava fino a questi ultimi tempi la dimostrazione della presenza di anticorpi circolanti nei casi di idiosincrasie per sostanze medicamentose a struttura chimica nota: ma, assai recentemente, il Koenigsfeld (1925, 2) ed il Biberstein (1926) han potuto dimostrare la loro presenza in alcuni casi di idiosincrasia per mercurio, salvarsan, bismuto, piramidone: sicché é superato anche quest' ultimo ostacolo che sembrava dovesse rimanere a distinguere queste forme dalle altre: ed appare sempre meno legittima una divisione basata sulla natura della sostanza anafilattizzante.

Vero é che si é notata qualche differenza fra l' azione di questi antigeni, talvolta forse non proteici, e gli antigeni classici, quale l' ovalbumina (Walzer e Grove, 1925): ma é noto da tempo che anche fra il potere antigeno delle diverse proteine possono sussistere differenze abbastanza profonde (vedi, p.e., le ricerche sui diversi antigeni contenuti nel siero, di Dale e Hartley, 1916; di Stern, 1922; e di Doerr e Berger, 1921, 1922); sicché alle differenze notate non può attribuirsi valore decisivo.

Più importanti possono forse apparire le differenze descritte a proposito della reazione dei nuovi anticorpi ai quali si é accennato sopra (reagine atopiche di Coca) con i relativi antigeni. Esse consistano essenzialmente: (*a*) nella possibilità di ottenere reazioni ripetute somministrando ripetutamente la stessa dose del relativo antigene (atopene di Coca) (Coca e Grove, 1925; Levine e Coca, 1926; Walzer, 1926), mentre nel caso degli anticorpi anafilattici noti fino ad oggi una seconda reazione può essere ottenuta soltanto somministrando un multiplo della dose usata per scatenare la prima reazione; (*b*) nella incapacità di questi anticorpi a neutralizzare il relativo antigene (Levine e Coca, 1926): ed infine (*c*) nella incapacità di questi anticorpi a sensibilizzare l' utero di cavia (Coca e Grove, 1925).

Nessuno di questi argomenti sembra però decisivo: ché da un lato, anche nell' esperimento d' anafilassi tipico, la stessa dose di questi particolari antigeni può determinare ripetutamente una reazione (Walzer e Grove, 1925) onde sembra trattarsi piuttosto di una particolarità del legame fra antigene ed anticorpo che non di una profonda diversità di questo: dall' altro, se queste reagine sono incapaci di sensibilizzare l' utero di cavia, non é meno vero che l' anticorpo anafilattico di coniglio non é capace di sensibilizzare la pelle umana (Coca e Grove, 1925): sicché sembra trattarsi piuttosto di una stretta specificità di anticorpi, che non di una più profonda diversità di natura. Infine, per quel che riguarda l' incapacità a neutralizzare l' antigene, dato il metodo di esperimento degli autori (saggio del potere scatenante delle miscele di reagina e atopene usate per iniezione intracutanea in individui resi passivamente ipersensibili), si può forse pensare ad una maggiore avidità delle reagine sessili, in vivo, rispetto a quelle circolanti nel sangue. Si può infatti ritenere che queste rappresentino delle sostanze in via di eliminazione, e come tali fisiologicamente meno attive: in questo senso depongono anche i risultati di Levine e Coca (1926) i quali non sono riusciti a dimostrare un parallelismo fra la gravità dei sintomi clinici e il contenuto del sangue in reagine, la presenza in circolo delle quali sarebbe perciò un fenomeno concomitante, ma non essenziale. Anche

qui, il parallelismo con l' anafilassi sperimentale é perfetto: ché anche in questa ultima manca spesso ogni rapporto fra il contenuto del sangue in anticorpi e la gravità delle reazioni che si possono ottenere.

In ogni modo, allo stato attuale delle ricerche, in pieno sviluppo lungo le linee ora accennate, non sembra legittimo cercare nella natura della sostanza anafilattizzante un criterio distintivo della anafilassi. Il futuro potrà forse mostrare che le differenze indicate sono tanto profonde da richiedere una netta distinzione fra l' anafilassi e queste altre forme di reazione fra antigene ed anticorpo: ma al momento attuale, quando é stata da poco tempo data la dimostrazione della presenza in queste forme di anticorpi circolanti, l' assenza dei quali aveva rappresentato fino ad oggi uno dei criteri più solidi di differenziazione, e la constatazione dei quali consente il loro inquadramento nel gruppo delle reazioni antigene-anticorpo, sembra che le ricerche procedano piuttosto nel senso di diminuire le differenze apparenti e di mostrare sempre più la fondamentale unità dei fenomeni in esame.

Occorre poi ricordare che i dati soprariferiti sono stati raccolti quasi esclusivamente da esperimenti sui mammiferi; nei quali soltanto l' anafilassi é stata studiata a fondo. Non sappiamo quindi fino a qual punto essi siano estensibili ad altri gruppi animali. Questo deve naturalmente renderci anche più cauti ed alieni da ogni tentativo di basare sulle qualità della sostanza provocatrice il criterio risolutivo di uno stato anafilattico: e ci deve anche fare ritenere non esatto il criterio, sul quale insiste il Doerr (1922), della necessità che un anafilattogeno, per esser considerato tale, sensibilizzi tutte le specie sensibili. L' ammetter ciò implica limitazioni ingiustificate che non possiamo accettare.

A conclusione, possiamo dire che, fermo restando come criterio distintivo unico dell' anafilassi quello dell' esistenza di una precedente sensibilizzazione, seguita da un periodo di incubazione, la diversità degli anafilattogeni potrà essere eventualmente invocata in futuro quale criterio di ulteriori suddivisioni.

(c) CARATTERI DELL' ANTICORPO ANAFILATTICO.

Abbiamo visto che lo studio delle sostanze anafilattizzanti non può fornirci un criterio definitivo per l' anafilassi. Resta a vedere se un simile criterio può essere fornito dall' anticorpo anafilattico.

Che un anticorpo anafilattico esista é fuori dubbio, fino dalle prime ricerche nelle quali Otto (1907) riuscì a dimostrare la trasmissibilità della anafilassi con il siero di un animale sensibilizzato: ma le opinioni sulla sua natura variano notevolmente. Mentre il Doerr sostiene sempre ed ancora la sua identità con le precipitine (Doerr, 1922; Doerr e Hallauer, 1926, 1927), l' Otto lo ritiene invece distinto da queste (Otto, 1924; Otto e Shirakawa, 1924; Otto ed Ornstein, 1926); e gli autori sono divisi fra le due dottrine.

Negli ultimi anni, come si é detto sopra, é stata dimostrata l' esistenza di anticorpi anche in molti casi nei quali essa non era fino ad oggi riuscita. Questi anticorpi sarebbero dotati di proprietà differenti da quelle descritte in precedenza per l' anticorpo anafilattico; sicché il Coca (1926) ritiene che essi possano servire a distinguere un gruppo di reazioni dall' altro. Ma da un lato, come si é detto sopra,

queste differenze non appaiono essenziali: dall' altro é noto che differenze profonde esistono, p.e., fra l' anticorpo anafilattico dei mammiferi e quello degli uccelli (Uhlenhuth e Haendel, 1909, 1910; Friedberger e Hartoch, 1909); sicché non sembra che una differenza nella natura degli anticorpi, anche sicuramente stabilita, possa da sola costituire un criterio distintivo, quando sussistano degli altri elementi per affermare l' esistenza di uno stato anafilattico.

(d) L' ANTIANAFILASSI.

Un altro elemento é stato spesso invocato a caratterizzare la reazione anafilattica rispetto ad altri tipi di reazione: l' esistenza, dopo lo choc, di un periodo durante il quale l' organismo, o meglio i suoi organi dello choc, sono incapaci di reagire ad una nuova introduzione dell' antigene. Come si é detto in precedenza, gli organi dello choc possono non essere quelli produttori dell' anticorpo: i quali ultimi sembrano risentire relativamente poco gli effetti dello choc dato che, almeno nella cavia, essi ricominciano ben presto ad inviare anticorpi in circolo. Dato questo fatto, sicuro almeno nella cavia, e dato che gli organi dello choc restano ugualmente sensibili alla reazione antigene-anticorpo, provocata con un altro antigene (salvo che in un primo periodo, quello della cosidetta antianafilassi non specifica, nel quale, per il fatto stesso del danneggiamento provocato dalla reazione anafilattica, é diminuita la capacità di reazione a qualunque stimolo), l' incapacità di reagire ad una nuova introduzione dello stesso antigene non si presenta di facile spiegazione. Si può forse pensare ad una incapacità delle cellule degli organi dello choc a fissare quei particolari anticorpi.

Negli ultimi tempi, e sempre a proposito dei nuovi anticorpi scoperti—le reagine atopiche—diversi autori americani (vedi sopra) hanno affermato che é possibile, ripetendo la somministrazione di dosi eguali dell' atopene, ottenere ripetute reazioni dalla stessa zona cutanea.

In questo caso quindi sembra che l' antianafilassi manchi, o sia tardiva. É stato però dimostrato che le sostanze antigene-polline impiegate in queste ricerche, anche nel classico esperimento anafilattico del Dale sull' utero di cavia possono provocare, ripetendo la stessa dose, contrazioni ripetute: sicché, come si é accennato sopra, é per lo meno possibile che il fenomeno sia dovuto alla particolare natura del legame fra antigene e anticorpo e non a diversità di questo. Si può, p.e., pensare che il legame fra la reagina e l' atopene sia assai labile: e che l' atopene possa facilmente esserne liberato, e passare nel sangue: sicché la reagina, fissata sulle cellule, possa di nuovo reagire colla nuova dose di atopene introdotta. Ma anche se così non fosse, questo fenomeno unico non basterebbe ad infirmare la natura anafilattica di quei fenomeni. Quanto all' antianafilassi, il giudizio sulla sua presenza costante o meno deve restare per ora in sospeso.

ANAFILASSI ED IMMUNITÀ.

L' analisi successiva dei diversi momenti del processo anafilattico e dei suoi diversi elementi ci ha dimostrato che a nessuno di questi si può attribuire un valore risolutivo, senza introdurre limitazioni arbitrarie del campo definito dal dato che

abbiamo posto a base del nostro esame: la comparsa cioé, in seguito ad un primo trattamento con una determinata sostanza e dopo un periodo determinato, della proprietà di dar luogo, in seguito ad una nuova introduzione di questa medesima sostanza, ad una reazione sempre ugale nello stesso animale, e che può variare da una specie all' altra.

Si é già visto in precedenza come il fenomeno si possa considerare perfettamente parallelo a quelli che si svolgono nel campo dell' immunità: ed, insieme a questi, sia espressione di una modificata reattività dell' organismo, l' allergia. La diversità del risultato finale, in un caso favorevole, nell' altro dannoso all' organismo ospite, non può certamente essere invocata per approfondire la differenza fra i due fenomeni e farne quasi dei contrapposti: nello stadio finale l' organismo funziona soltanto da indicatore della reazione fra antigene ed anticorpo: e non ha perciò alcuna importanza se l' effetto della reazione sia ad esso dannoso o favorevole. In un certo senso, questo risultato finale é puramente casuale e non riguarda il meccanismo della sensibilizzazione, che é poi l' unico che realmente c' interessi. In ambedue i casi avviene, secondo la terminologia Ehrlichiana, una neutralizzazione dell' antigene da parte dell' anticorpo, come é dimostrato dal fatto che, quando si tratti di sostanze primitivamente attive, manca la loro azione specifica. In ambedue i casi quindi si stabilisce una identica condizione di immunità rispetto a questa azione specifica; condizione che può o no accompagnarsi alla comparsa di fenomeni anafilattici, la quale come si vede, rappresenta un fenomeno puramente finale, conclusivo, ripetiamo casuale, nel senso che non deriva necessariamente dalla sequela di fenomeni scatenati dalla prima introduzione dell' antigene. É in questi casi che alcuni autori parlano di coesistenza dell' anafilassi con l' immunità; espressione inesatta, o quanto meno atta a generare equivoci; dato che non si tratta di due fenomeni che si producono contemporaneamente, ma di due aspetti dello stesso fenomeno, e cioé della reazione fra antigene e anticorpo.

In un altro punto tuttavia é possibile scoprire una più sottile differenza fra immunità ed anafilassi. Mentre gli organismi viventi si vengono a trovare abbastanza facilmente in condizioni tali da favorire la produzione di fenomeni immunitari, assai più difficili a verificarsi sono le condizioni necessarie allo stabilirsi dell' anafilassi. Se se ne tolgono alcune forme morbose, esclusivamente umane, quali l' asma da fieno, etc., i fenomeni anafilattici sono fenomeni sperimentali, e che soltanto l' esperimento é riuscito a rivelare prima, a ripetere poi. Come dice il Dale (1920) con una felice espressione, "a generalised anaphylactic reaction, the anaphylactic shock, is a creation of the injection needle," la reazione anafilattica generale, lo choc anafilattico, é una creazione dell' ago da iniezione. In questo senso essi rappresentano quasi un unicum nel campo delle ricerche biologiche; la provocazione, cioé, nell' organismo integro, con un intervento quasi insignificante, della comparsa di proprietà prima inesistenti o esistenti solo allo stato potenziale. La regolarità delle risposte che si ottengono é perciò in tanto più notevole, in quanto in questo campo non può aver agito alcun elemento selettore o eguagliatore: e la completezza e la perfezione con la quale il meccanismo delle reazioni anafilattiche ci appare ci obbliga a ritenere che esso si basi su un substrato ben altrimenti solido

che non quello semplicemente immunitario, la chiamata in causa del quale ha
dovuto, nel corso delle generazioni, essere soltanto saltuaria. Dobbiamo perciò
ammettere che il meccanismo delle reazioni anafilattiche, ed in genere i meccanismi
immunitari, utilizzino degli altri meccanismi preesistenti ed assai perfezionati, che
soli possono spiegare la complicatezza ed insieme la perfetta regolarità di una
risposta che l' organismo é chiamato a dare così di rado; si può pensare, p.e., ai
meccanismi di assunzione delle sostanze alimentari nell' interno delle cellule.

Se é così, e se in effetto la reazione anafilattica e le reazioni immunitarie in
genere rappresentano particolari modalità di reazione di meccanismi interessati ad
alcuni fenomeni fondamentali della cellula, la loro diffusione deve essere assai
ampia, anzi generale; e deve essere possibile provocarli in tutti gli organismi viventi.
É compito delle prossime pagine esaminare se e fino a qual punto questo assunto
risulti verificato dalle indagini fino ad oggi compiute.

L' ANAFILASSI NEI DIVERSI GRUPPI.

Le ricerche rivolte a dimostrare l' esistenza di fenomeni immunitari in animali
diversi dai mammiferi, nei quali essi sono stati primitivamente scoperti e studiati,
non sono scarse né tutte recenti. Dal Metchnikow (1897) al Dungern (1903), al
Noguchi (1903), alla Szczawinska (1905), al Fredericq (1910), al Drew (1911) e fino
ai più recenti, Cantacuzène (1923), Metalnikow (passim), Paillot (passim) ed altri,
molti autori hanno studiato i fenomeni immunitari e la produzione degli anticorpi
nei diversi gruppi; e non é compito di questo lavoro di procedere ad una rivista dei
risultati ottenuti. Solo si può ricordare, sintetizzando, che la maggior parte di
questi autori non é riuscita a dimostrare la presenza nel sangue degli invertebrati,
dopo l' immunizzazione, dei più comuni anticorpi: senza per altro che questo risul-
tato autorizzi a concludere per la incapacità dei detti animali a produrre anticorpi,
i quali potrebbero, come si é detto, non comparire nel sangue e restar sempre
sessili. Il problema non può esser risolto definitivamente se non coll' ausilio della
reazione anafilattica, la quale, trasportando il luogo dell' incontro e della reazione
dell' anticorpo e dell' antigene dalle nostre provette nell' intimità dei tessuti, é sola
in grado di rivelare la presenza anche di anticorpi permanentemente sessili, che non
compaiono mai nel sangue.

Gli esperimenti di anafilassi però non sono stati eseguiti che in un numero
relativamente esiguo di specie, che passeremo rapidamente in rassegna.

(a) MAMMIFERI.

Il maggior numero degli esperimenti sono stati compiuti sulla cavia, la quale, fra
i comuni animali di laboratorio, sembra quello meglio adatto a questo genere di
ricerche. É sulla cavia che sono stati ottenuti quasi tutti i risultati che hanno poi
dato origine alle diverse teorie: ma, come ben fa notare il Doerr (1922), non é detto
che questo fatto sia sempre stato favorevole al successivo sviluppo delle ricerche.
L' organo dello choc é nella cavia rappresentato dai bronchioli[1].

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[1] Per la descrizione e l' analisi dello choc nella cavia e negli altri mammiferi, vedi Doerr, R. in
Weichardt's Ergebnisse der Hygiene Bakteriologie, etc., V, 1922, 175 e seg.

Altre specie nelle quali le conoscenze sono abbastanza approfondite sono il coniglio (nel quale l' organo dello choc é rappresentato dalle arteriole polmonari), il cane (organo dello choc: fegato), e, sebbene in grado minore, il gatto, nel quale l' apparecchio circolatorio partecipa primitivamente allo choc.

Assai meno profonde sono le nostre conoscenze sull' anafilassi degli altri mammiferi. Fra gli animali di laboratorio molte ricerche sono state compiute sul topo bianco e sul ratto bianco: i risultati degli autori più antichi furono incerti o negativi, e furono il Ritz (1911) e subito dopo il Sarnowsky (1913) i primi a stabilire con certezza la possibilità di sensibilizzare i topi bianchi. Nel ratto Novy e de Kruif (1917) ottennero solo delle reazioni poco intense e poco nette. Negli ultimi anni poi i Parker (1924) riuscirono ad ottenere risultati positivi per quel che riguarda l' anafilassi attiva e passiva del ratto bianco: mentre Longcope (1922), Spain e Grove (1925), ed Ebert (1927) negli stessi animali ebbero risultati negativi. Le divergenze fra questi diversi autori trovano forse la loro spiegazione nei risultati del Flashman (1926) che vide che nel ratto bianco lo choc anafilattico si presenta soltanto dopo la estirpazione dei surreni. Recentemente infine Friedberger e Seydenberg (1917) hanno potuto dimostrare l' anafilassi nel preparato vasale di ratto. Anche recentemente Schiemann e Meyer (1926) tentarono con successo il trasporto omologo ed eterologo dell' anafilassi nel topo bianco[1].

Fenomeni anafilattici sono stati inoltre dimostrati nei cavalli, nei buoi, nelle capre, nei montoni, nei maiali (vedi per l' elenco degli autori che hanno lavorato sulle diverse specie: Doerr, 1913; ed inoltre Skiba (1913), per l' anafilassi dei bovini; Roos (1915), e Groth (1925), per quella del maiale; Ritzenthaler (1924), per quella del cavallo); e nell' opossum (Edmunds, 1914).

Uhlenhuth e Haendel (1910) e Yamanouchi (1910) non riuscirono a sensibilizzare con siero di cavallo una scimmia, Macacus rhesus. Uhlenhuth e Haendel riuscirono però a sensibilizzare passivamente la cavia con il siero di questa stessa scimmia, apparentemente non anafilattica. Anche l' Auer ottenne risultati negativi nelle scimmie; e lo Zinsser (1922), sperimentando su diverse specie di Macacus, ottenne alcuni deboli risultati positivi, che rammentavano la malattia da siero dell' uomo.

Quanto all' uomo le condizioni sono alquanto più complicate: e se é certamente possibile la sensibilizzazione sperimentale nelle condizioni più tipiche e probative, la presenza in clinica di fenomeni di carattere anafilattico, la produzione dei quali non si può naturalmente svolgere tutta nell' ambito della nostra osservazione, é atta ad oscurare il quadro e a renderne più difficile la interpretazione. Pur tuttavia, come si é detto, esistono, specie nella letteratura più recente, casi nei quali la sensibilizzazione é avvenuta in via assolutamente sperimentale e con tutti i possibili controlli (v.p.e. Köhler e Heilmann, 1924); sicché la possibilità di ottenere nell' uomo la anafilassi é posta fuori di dubbio: ed appare più legittimo riportare a questa fe-

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[1] Questi due autori hanno trovato che, anche dopo lo choc da anafilassi passiva, in questi animali il periodo di antianafilassi é breve, come il Sarnowsky aveva dimostrato dopo lo choc da anafilassi attiva. Essi pensano alla possibilità che in qualche caso la ricomparsa della sensibilità sia dovuta ad una sensibilizzazione attiva da parte dell' antigene iniettato per provocare lo choc; ma sembra che essi ignorino la dimostrazione di questo fatto data dallo scrivente nella cavia (Sereni, 1924, 1925).

nomeni clinici i quali, per essersi svolti in condizioni meno perfette, possono dar luogo a dubbi. Un' ulteriore prova in questo senso é stata portata dalla dimostrazione, data dal Prausnitz e Küster, e già più volte ricordata, della possibilità di sensibilizzare passivamente per una determinata sostanza la cute di un individuo sano con la iniezione intracutanea del siero di un individuo sensibile a quella stessa sostanza.

Con questa prova della trasmissione passiva della sensibilità il circolo sembra chiuso: e le differenze notate, come si é già detto più volte, non sembrano tali da legittimare una divisione.

In conclusione si può affermare che tra i mammiferi l' anafilassi é stata constata*a in tutte le specie nelle quali é stata ricercata: e il numero di queste sembra abbastanza largo perché il fenomeno possa ritenersi generale.

(b) Uccelli.

Fra gli uccelli già Friedberger e Hartoch (1909) riuscirono ad anafilattizzare attivamente e passivamente il colombo e l' anatra, e, con qualche difficoltà, il pollo. Subito dopo Uhlenhuth e Haendel (1910) riuscirono ad anafilattizzare gli stessi animali, salvo il pollo, ed in più anche l' oca, la quale anzi, fra gli uccelli, sarebbe il più adatto a queste ricerche. Anche Arthus ha ottenuto l' anafilattizzazione dei piccioni e delle anatre, ed Arloing e Langeron (1923) quella dei piccioni. In epoca più recente, il Detre (1926) ha ripreso in esame la questione ed ha potuto constatare che negli uccelli (colombi) il periodo di incubazione é assai più breve (comparsa della sensibilità al 6°–7° giorno, massimo al 9°–11° giorno): ed ha messo questo fatto in relazione con la più elevata temperatura interna. I suoi dati sono confermati da quelli del Gahringer (1926), che ha visto iniziarsi la ipersensibilità già al 4° giorno, per raggiungere il massimo al 12°, e scomparire fra il 16° e il 17°. Questo autore ha potuto constatare anche un periodo di antianafilassi dopo lo choc. L' Eds infine (1926) ha potuto anch' egli constatare nel piccione l' esistenza di uno stato anafilattico, che Hanzlik e Stockton (1926) e Hanzlik, Butt e Stockton (1927) sono riusciti a dimostrare elegantemente, registrando in vivo e nell' animale intatto la contrazione dei muscoli dell' ingluvie.

Anche negli uccelli, come si vede, l' anafilassi sembra essere un fenomeno generale: pur se sarebbe desiderabile l' estensione dell' esperimento ad altre specie.

(c) Vertebrati pecilotermi.

Le condizioni sono assai diverse per le altre classi dei vertebrati: le ricerche sono state fino ad oggi limitate quasi esclusivamente alla rana, nonostante l' interesse notevole che queste ricerche possono avere da molti punti di vista. Friedberger e Mita (1911) furono i primi a dimostrare la possibilità di anafilattizzare le rane, ma solo quelle di estate e non quelle d' inverno; ed a constatare come in questi animali i fenomeni si svolgessero essenzialmente a carico del cuore. Le loro ricerche sono state poi ripetute, con diverse modalità, da parecchi autori. Fröhlich (1914) poté confermare pienamente i loro risultati: e vide inoltre che anche nella rana si possono ottenere reazioni locali, corrispondenti al ben noto fenomeno di Arthus. Arnoldi e Leschke (1920) videro che nel preparato di Läwen-Trendelenburg di rana sensi-

bilizzata l' aggiunta del siero antigene provoca una vaso-dilatazione invece che una
vaso-costrizione, come nel normale. Kochmann e Schmidt (1922) non poterono
confermare questi risultati: e neppure quelli del Friedberger e Mita sul cuore.
Arloing e Langeron (1923, 2) ottennero risultati negativi nella rana ed in diverse
specie di pesci di acqua dolce. In epoca più recente il Koenigsfeld (1925) ha
ottenuto risultati positivi sul cuore di rana: come il Goodner (1926), il quale invece
non ottenne risultati sperimentando con l' animale intero o con la muscolatura liscia
del tratto digerente. Risultati negativi ottennero pure Kritchewsky e Birger (1924)
e la Skarzynska (1925) (ma i risultati di quest' ultima hanno scarso valore, perché
ella ha sperimentato sulle rane d' inverno); positivi invece Friede e Ebert (1927); e
dopo di loro, e con una tecnica originale, che dovrebbe escludere l' intervento nello
choc di fenomeni umorali e stabilire definitivamente la localizzazione della reazione
nelle cellule, Kritchewsky e Friede (1927). Come si vede, i risultati affermano
nettamente la possibilità di ottenere la sensibilizzazione della rana: i risultati
negativi sono probabilmente dovuti a deficienze tecniche, e stanno ad indicare
alcune interessanti direzioni di ricerca: quella, p.e., sulla possibile influenza della
temperatura sul processo della sensibilizzazione, chiaramente messa in luce dai
risultati negativi di Friedberger e Castelli e della Skarzynska sulle rane d' inverno.

(d) INVERTEBRATI.

Se le ricerche sull' anafilassi dei vertebrati eterotermi si limitano a quelle ora
citate, anche più scarsa, quasi nulla, é la messe nel campo vastissimo degli inverte-
brati. Non mi sono note che le ricerche del Metalnikow (1922), il quale non riuscì
a dimostrare (dopo 14–15 giorni dalla prima iniezione) una anafilassi del bruco di
Galleria melonella per diversi sieri. In un altro bruco invece, quello di Cnethocampa
pityocampa, egli riuscì, dopo un primo trattamento con vibrioni colerici, a dimo-
strare che, mentre era aumentata la resistenza alle piccole dosi, era diminuita quella
alle dosi più grandi, che provocavano la morte più rapidamente e con un quadro
diverso dal normale.

La scarsità delle ricerche sui vertebrati eterotermi e l' assenza o quasi di ricerche
sugli invertebrati mi ha indotto recentemente a riprendere l' esame sperimentale
della questione. Ho istituito perciò ricerche su animali di diversi gruppi: ed ho già
ottenuto risultati che mi permettono di affermare con certezza la possibilità di
ottenere la sensibilizzazione dei selaci (Scyllium canicula) e dei cefalopodi (Octopus
vulgaris, Eledone moschata). Risultati promettenti, ma non ancora definitivi, ho
ottenuto anche su un teleosteo (Crenilabrus pavo), su un tunicato (Cyona intesti-
nalis), su un crostaceo (Maja verrucosa), e su un gefireo (Sipunculus nudus). Non
é questo il luogo per descrivere minutamente i fenomeni, naturalmente diversi,
ottenuti in ciascuna di queste specie; basti però affermare che in tutte le iniezioni
successive dell' antigene diedero origine a fenomeni assai più gravi della prima, di
solito innocua. Si può perciò affermare che l' anafilassi può esser dimostrata, con
adatti accorgimenti, in animali scelti a caso in molti gruppi, ed anche in quegli
animali nei quali le ricerche in vitro non sono riuscite a rivelare la comparsa di
anticorpi nel sangue dopo l' immunizzazione.

(e) Piante.

Negli ultimi anni, il campo della anafilassi ha subito un notevole ampliamento, perché si é tentato di dimostrare fenomeni analoghi anche nel regno vegetale. Furono dapprima Lumière e Couturier (1921) a dimostrare che parecchie piante (acetosella, giacinto, cipolla) appassivano dopo una seconda iniezione di siero praticata a 15–30 giorni di distanza dalla prima. Disgraziatamente, le loro ricerche non sono state confermate dagli autori che le hanno ripetute (Novoa Santos e Gonzalez Criado, 1924; Longo e Cesaris-Demel, 1925).

(f) Batterii.

Ben altrimenti importanti ed interessanti si presentano le numerose ricerche eseguite da diversi autori francesi e tedeschi su fenomeni di anafilassi nei batterii. In Francia il Richet, con diversi collaboratori (Richet, 1921; Richet, Bachrach e Cardot, 1921; Bachrach, 1926) ha studiato gli effetti sul bacillo lattico di piccole dosi di diversi sali, bicloruro di mercurio, nitrato e solfato di tallio, arseniato di potassio, etc., ed ha visto che dosi minime di questi sali, tali da influenzare appena l' accrescimento dei batterii, sensibilizzano questi ultimi per le dosi maggiori, che riescono poi assai più dannose che nei ceppi normali. Il fenomeno é intimamente connesso con i fenomeni di adattamento, che si ottengono però di solito con dosi maggiori, e in un periodo di tempo più lungo. In altri casi invece, e specialmente col sublimato, si ha in un primo tempo l' adattamento, al quale succede più tardi la sensibilizzazione. Questa sensibilizzazione secondaria si ottiene anche con il nitrato di tallio, ma é assai tardiva. La Bachrach (1926) ha dimostrato che in qualche caso la sensibilizzazione può essere simulata da uno spostamento dell' optimum termico dei batterii: ma in altri casi invece la sensibilizzazione sussiste realmente.

Contemporanee a queste ricerche del Richet e della sua scuola, sono le ricerche compiute dallo Schnabel con diversi allievi (Schnabel, 1922, 1923, 1924; Schnabel e Kasarnowsky, 1923, 1924; Iungeblut, 1923; Hayaishi, 1924), sui fenomeni di ipersensibilità provocati in vari germi da varie sostanze tossiche (optochina, chinina, tripaflavina, nitrato d' argento, sublimato, fenolo, formaldeide). Questi autori hanno impiegato per la dimostrazione non già il metodo delle colture successive, ma quello della riduzione del bleu di metilene, il quale, dando risultati quasi immediati, fotografa le condizioni della coltura in un determinato momento, e si sottrae perciò alle cause d' errore connesse col metodo delle colture, ed una delle quali é stata messa in luce dalla Bachrach. Con questo metodo, lo Schnabel ed i suoi collaboratori hanno potuto anch' essi dimostrare che sono le piccolissime dosi della sostanza tossica quelle che agiscono sensibilizzando, mentre le dosi più grandi aumentano la resistenza del germe. Anch' egli ha potuto constatare la grande importanza del fattore tempo, ed ha visto succedere alla ipersensibilità l' adattamento e l' aumento della resistenza. L' ipersensibilità é specifica; soltanto quando essa é di grado notevole si estende anche a sostanze affini per struttura chimica (optochina e chinina), o, probabilmente, per il modo d' azione (nitrato d' argento e sublimato). L' ipersensibilizzazione dei batteri é possibile non soltanto nelle colture ma anche nell' organismo infetto. Infine lo Schnabel é riuscito a dimostrare che il filtrato di colture

di batteri resi resistenti, p.e., all' optochina od al sublimato, é capace di conferire una certa resistenza alle stesse sostanze anche ad altre colture: e per quanto egli non abbia ancora pubblicato risultati analoghi a proposito dei fenomeni di ipersensibilità, é probabile che anche per questi la trasmissibilità possa essere dimostrata.

I risultati in gran parte concordanti di queste due scuole a proposito dei fenomeni di ipersensibilità dei batteri hanno importanza non soltanto per il fatto che essi estendono ulteriormente il campo degli esseri nei quali questi fenomeni sono stati constatati: ma anche perché essi offrono alla nostra considerazione alcuni elementi nuovi. Da un lato, essi confermano la possibilità, sempre riaffermata e sempre nuovamente negata a proposito degli organismi superiori, di ottenere l' anafilassi con sostanze a struttura chimica nota e relativamente semplice. Anche se questa possibilità fosse limitata ai batteri, essa basterebbe a distruggere la legittimità di ogni esclusione basata sulla natura dell' antigene. Mancano, o mi sono ignote, ricerche tendenti a rivelare la possibilità di sensibilizzare i batteri per sostanze chimiche più complesse ed in particolar modo per le proteine, la natura antigene delle quali é così ben nota dalle ricerche sugli animali superiori. Le ricerche di Arloing e Thévenot (1922) con terreni al siero di cavallo, che potrebbero essere interpretate in questo senso, non sono del tutto chiare ed univoche. Vero é che sia il Richet (1921) che lo Schnabel (1924) tendono a riportare il primo l' attenuazione progressiva delle colture coltivate sullo stesso terreno, il secondo lo stesso fenomeno del d'Herelle, a fenomeni di sensibilizzazione per le sostanze prodotte dal metabolismo dei batteri stessi. Anche il Gohs (1926) ha recentemente sostenuto che il fenomeno del d'Herelle deve essere considerato come un fenomeno di anafilassi nei batteri.

Si tratta però ancora di pure ipotesi; e d' altra parte non sembra impossibile di ammettere che la natura chimica degli anafilattogeni possa variare da un gruppo all' altro, parallelamente forse alla natura del ricambio. A questo proposito sarebbe interessante ripetere i tentativi di sensibilizzazione delle piante superiori con sostanze chimiche più semplici; p.e., con sali.

É interessante notare, a proposito dell' anafilassi dei batteri, con quanta facilità la storia, anche scientifica, segua vie già precedentemente battute. Anche in questo caso infatti, come abbiamo visto essere avvenuto per l' anafilassi dei mammiferi, le prime (e fino ad oggi le sole) ricerche sono state compiute con sostanze primitivamente tossiche.

Ma da un altro punto di vista le ricerche sulla anafilassi dei batteri hanno un grande interesse generale: in quanto, cioé, esse pongono in luce il problema della eredità nei rapporti dell' anafilassi. É evidente infatti che, nel caso dei batteri, quelli sui quali noi sperimentiamo l' effetto della coltura in ambiente tossico non sono gli stessi sui quali la sostanza ha agito, ma i loro discendenti, sui quali la sostanza tossica non può avere agito direttamente, perché quasi sempre, fra la coltura sensibilizzante e la coltura di prova, gli Aa. hanno intercalato una coltura in terreno del tutto privo della sostanza tossica (corrispondente perciò al periodo di incubazione). Ci troviamo qui dinanzi al problema della eredità dei caratteri acquisiti, in tutta la sua imponenza e molteplicità: e naturalmente non é questo il momento per discuterlo. Interessa a noi soltanto constatare come l' eredità possa venire a complicare

i fenomeni dell' anafilassi e dell' immunità; e ci sembra che questo debba condurre ad abbandonare la posizione, p.e., del Coca, il quale pone la parziale ereditarietà di alcuni fenomeni di ipersensibilità dell' uomo come uno dei criteri che ne impongono la separazione dai fenomeni anafilattici. É pacifico che questi non sono, nella loro manifestazione più classica, nella cavia, per nulla ereditari : ma la constatazione sicura del gioco di meccanismi ereditari nell' anafilassi di gruppi anche lontanissimi deve render assai cauti prima di considerare questo elemento come criterio di netta e precisa distinzione. Occorre pure ricordare che, nel caso dell' uomo, non si tratta quasi mai di una eredità identica : non si eredita cioè, o solo di rado, la ipersensibilità ad una determinata sostanza, ma la disposizione generica alla ipersensibilità : per usare i termini del Coca, si eredita uno stato di particolare reattività dell' organo produttore delle reagine atopiche. L' elevare però l' elemento della maggiore disposizione a produrre reagine a criterio di distinzione fra l' anafilassi e le altre forme della sensibilità implica l' ammissione che, nei riguardi dell' anafilassi, tutti gli individui di una specie abbiano la stessa capacità di reazione. Che questo non sia, era già dimostrato, p.e., dalle antiche ricerche del Vasconcellos (1907), il quale, sperimentando su cavie brasiliane, trovò che queste molto più difficilmente presentano uno choc mortale : ed é cosa nota in tutti i laboratori che, negli esperimenti sull' anafilassi, le cavie a pelo lungo e arruffato danno risultati diversi da quelli che si ottengono nelle cavie comuni. Recentemente poi, Lewis e Loomis (1925), sperimentando con razze pure di cavie, hanno potuto riconoscere che le reazioni anafilattiche ottenibili nei diversi stipiti possono variare assai per intensità : e che la suscettibilità alla anafilassi varia parallelamente a quella per la tubercolosi. A proposito di quest' ultima poi, gli stessi autori hanno potuto dimostrare che la eredità della suscettibilità si verifica almeno in parte secondo le regole Mendeliane. Questi risultati possono esser messi in relazione con quelli, p.e., di Coca, Deibert e Menger, che hanno potuto dimostrare che la malattia da siero, l' asma, etc., sono assai meno frequenti fra gli Indiani d' America che fra i bianchi : e d' altra parte con quelli dell' Ancona, giustamente valorizzati dal Doerr, i quali dimostrano che, in condizioni opportune, quasi tutti gli individui umani sono in grado di acquistare la ipersensibilità per una determinata sostanza. L' anello sembra perciò chiuso : il fattore ereditario e costituzionale, che sembrava finora trascurabile nell' anafilassi sperimentale, perché la maggior parte degli esperimenti era stata eseguita sulla cavia, animale eccessivamente disposto alla sensibilizzazione, é nuovamente posto in luce ; anche da questo punto di vista, appare chiara la fondamentale unità di tutti i fenomeni considerati : e la preminenza dell' uno o dell' altro aspetto può essere più argomento di una ulteriore classificazione, che di una divisione iniziale.

L' ANAFILASSI NEI DIVERSI PERIODI DELLA VITA INDIVIDUALE.

Quello che siam venuti riportando dalla letteratura nelle pagine precedenti dimostra che l' anafilassi é un fenomeno assai diffuso fra gli organismi viventi, anche se l' insufficiente estensione degli esperimenti consiglia una certa prudenza prima di procedere ad una generalizzazione. Resta a vedere in quale momento della ontogenesi compaia la capacità della reazione anafilattica.

Esistono a questo proposito delle ricerche, già antiche, del Friedberger e del Simmel (1913), che dimostrano che, mentre, nell' esperimento dell' anafilassi passiva, le cavie neonate sono sensibili come, o poco meno, degli adulti, nell' anafilassi attiva esse reagiscono assai meno intensamente: probabilmente perché é in esse meno intensa la produzione dell' anticorpo.

Negli ultimi anni poi, in una serie di ricerche in parte ancora in via di esecuzione (Sereni, 1925, 1926), ho potuto dimostrare che il feto di cavia può essere anafilattizzato (con una tecnica descritta altrove) già in un' epoca molto precoce del suo sviluppo intra-uterino, senza che contemporaneamente venga ad essere sensibilizzata attivamente la madre, la quale invece é anafilattizzata passivamente dagli anticorpi fetali.

Con queste ricerche é fornita la dimostrazione della precocità della comparsa del potere di produzione degli anticorpi: e l' anafilassi acquista un nuovo titolo per aspirare a quel carattere di generalità che abbiamo visto esser possibile attribuirle.

L' ANAFILASSI NEI DIVERSI ORGANI.

Dopo averne studiato l' apparizione nei diversi organismi ed, in un determinato organismo, il momento della comparsa, resta ancora a ricercare quali siano, in un organismo colpito, gli organi e i tessuti che partecipano al fenomeno. Come si é detto sopra, l' organo o il tessuto dello choc, nei diversi animali, non rappresentano se non il luogo nel quale la reazione é più appariscente e violenta, e più gravida di conseguenze: e la loro presenza non esclude che la reazione si possa svolgere ovunque. Così, p.e., mentre nella cavia l' organo dello choc é rappresentato dai bronchioli, ogni qualvolta é intervenuta la contrazione di questi, si può constatare che anche l' utero si trova in antianafilassi (Alexander, 1926). Questo dimostra che, per quanto non se ne siano constatati indizii, anche l' utero ha subito uno choc.

Il problema é distinto in due parti: una prima cioé che considera la capacità dei diversi organi e tessuti a rispondere alla reazione antigene-anticorpo, una seconda riguardante il luogo di produzione degli anticorpi.

Quanto alla prima, non vi possono essere dubbii: le ricerche di moltissimi autori dimostrano che quasi tutti i tessuti possono reagire.

Dopo le prime classiche ricerche dello Schultz (1910) e del Dale (1912), precedute per vero da quelle del Friedberger e Mita (1911) sul cuore di rana, che rimasero però relativamente sconosciute, é stata dimostrata, di solito nella cavia, la reazione dell' intestino (Massini, 1918); del cuore (Cesaris-Demel, 1910, 1912; nel coniglio); dei vasi sanguigni (Arnoldi e Leschke, 1920; Nakazawa, 1925; Friedberger e Seydenberg, 1926); della pelle, da moltissimi autori; del sistema nervoso centrale (Hashimoto, 1915; Spiegel e Kubo, 1923) e periferico (Yamanouchi, 1909; Kling, 1912; Nakazawa, 1925); del fegato (Hashimoto e Pick, 1913, 1914; e Manwaring); dei leucociti (Metalnikow, 1922); del condotto deferente (ricerche inedite dell' autore).

A proposito della teoria generale dell' anafilassi, é interessante notare che le manifestazioni presentate dai diversi organi nello choc non sono necessariamente nel senso di una diminuzione dell' attività, di un danneggiamento: ma spesso

anzi nel senso di una stimolazione, di un eccitamento: sicché appare ancora una volta quanto sia fallace la considerazione, basata esclusivamente sull' osservazione clinica grossolana, che fa dell' anafilassi qualcosa di necessariamente dannoso.

I risultati riferiti ci dicono però soltanto che tutti gli organi sopraindicati sono capaci di rispondere alla reazione antigene-anticorpo: o, se si vuole, che essi sono capaci di fissare l' anticorpo circolante. Nulla però questi risultati ci possono dire per quel che riguarda il secondo aspetto del problema: e cioé se le cellule di questi organi sono primitivamente modificate per il fatto della sensibilizzazione, sono cioé esse stesse capaci di produrre anticorpi. Il problema, come si é detto, si identifica con quello del luogo di produzione degli anticorpi anafilattici: e non appare perciò ancora del tutto risolto. In linea generale sembra che questi, come gli altri anticorpi, siano prodotti essenzialmente negli organi ematopoietici (Sereni, 1924, 1925): ma occorre riconoscere che le prove fornite, se tutte permettono di concludere per una formazione di anticorpi negli organi ematopoietici, non sono tali da escludere una formazione negli altri organi. La questione si deve perciò ritenere ancora allo studio: e, nell' attesa di una soluzione, non si può decidere se, negli organismi superiori, la produzione degli anticorpi é divenuta appannaggio di organi e cellule speciali o se é invece rimasto patrimonio di tutte le cellule.

CONCLUSIONE.

La sintesi che abbiamo tentata non é ancor oggi possibile e completa in ogni sua parte. La concezione dell' anafilassi come una modalità di reazione generale degli organismi viventi, in ogni loro specie, in ogni loro momento, ed in ogni loro parte, si basa ancora oggi su un materiale sperimentale che, se in alcune parti può apparire sufficiente e definitivo, in altre é ancora incompleto o assente. In ogni modo essa appare adatta ad inquadrare la maggior parte dei fenomeni già noti; e la constatazione avvenuta nelle pagine precedenti di tante e così notevoli coincidenze rende inutile ed inadeguato qualunque tentativo di distaccare dal gruppo dei fenomeni anafilattici questo o quel quadro sintomatico, che per qualche particolare non trovi riscontro in quelli già noti. Allo stato delle cose, qualunque fenomeno che soddisfi alle esigenze che abbiamo fissato in principio—modificazione della reattività susseguente ad un primo contatto, che si verifica soltanto dopo un periodo di incubazione, ed é dimostrabile soltanto con un rinnovato contatto con la medesima sostanza—deve essere considerato come un fenomeno anafilattico. Le dette condizioni sono perciò quelle necessarie e sufficienti a definire l' anafilassi, con il sottinteso che la modificazione della reattività si rivela di solito in senso sfavorevole all' organismo. Ma occorre tener sempre presente che attraverso questo sottinteso fa di nuovo capolino quel principio teleologico che abbiamo costantemente cercato di tener lontano: e che l' unità dei fenomeni di anafilassi e di immunità é veramente inscindibile, sicché gli uni continuamente si confondono e si accavallano con gli altri, e il risultato finale sembra bene spesso regolato dal caso.

L' indole di questo articolo, destinato prevalentemente a biologi, non ci consente di discutere quì particolarmente le diverse classificazioni possibili nel campo stesso dell' anafilassi; ma un così rapido mutare ed aumentare di dati sperimentali (si pensi

soltanto che nella sua ultima rassegna il Coca (1926) deve riconoscere che tutti gli argomenti da lui originalmente—sei anni prima—portati a favore della distinzione fra anafilassi ed atopia sono caduti o modificati) ci sembra debba convincere che non é ancora venuta l' ora di queste ulteriori suddivisioni. Che differenze debbano esistere fra le reazioni dei diversi organismi ci sembra a priori probabile: ché differenze simili esistono per tutti i fenomeni biologici. Ma, come noi parliamo sempre di respirazione, in qualunque modo essa avvenga, per polmoni, per branchie, per trachee, etc., così ci sembra poco logico di costruire delle barriere fra fenomeni che non differiscono certo tra loro più di quelli indicati, e che tutti rispondono alle condizioni poste in precedenza, e che sole derivano necessariamente dalla definizione stessa del fenomeno. Per il momento quindi non si può parlare che di questo in generale: riservando invece ad un futuro più o meno lontano le ulteriori suddivisioni, senza dubbio possibili.

Neppure é questo il luogo per discutere della pertinenza o meno allo anafilassi di alcuni fenomeni, quali, per esempio, la reazione alla tubercolina, dei quali da lungo tempo é in dubbio la posizione. Soltanto é forse opportuno ricordare—più veramente a proposito di altri esempi che di quello portato—che troppo spesso forse si é dimenticata la possibilità che, fra le infinite combinazioni, esistano nell' organismo anche quelle capaci di reagire con la sostanza introdotta. Che queste coincidenze, teoricamente possibili, non siano neppur troppo rare nella pratica é dimostrato, p.e., dai fenomeni della formazione eterogenetica di emolisine per le emazie di montone, descritti dal Forssmann. Naturalmente, in questi casi di coincidenze casuali, non si può parlare di anafilassi: come non é lecito parlare propriamente di anticorpi a proposito delle agglutinine, emolisine, etc., che sono state trovate da diversi autori, p.e., in molte piante: nei quali casi si tratta di proprietà non indotte dall' azione dei rispettivi antigeni e solo casualmente coincidenti con quelle che siamo abituati ad indicare con quei nomi.

L' ANAFILASSI COME METODO DI INDAGINE.

Se fino a questo momento abbiamo considerato l' anafilassi in sé, per l' importanza che essa può avere come fenomeno biologico generale, é opportuno da ultimo far seguire qualche breve considerazione sull' utilizzabilità e l' applicabilità dell' anafilassi come metodo d' indagine biologica in varii campi.

Come per le altre reazioni immunitarie, così per l' anafilassi, l' Uhlenhuth fu il primo (Uhlenhuth e Haendel, 1910) a tentarne l' applicazione a scopi biologici. Egli stabilì che, rispetto alla precipitazione, accanto ad alcuni svantaggi, l' esperimento della anafilassi presentava anche alcuni vantaggi, quali, p.e., la possibilità di impiego anche in casi nei quali la precipitazione non é applicabile. Egli compì anche alcuni esperimenti di sensibilizzazione con diversi secreti ed escreti (latte, succo gastrico, bile, urina, sudore), e vide che costantemente questi sensibilizzavano anche per il siero della stessa specie. Con l' esperimento dell' anafilassi, egli studiò anche i rapporti tra specie animali vicine, e riuscì così a distinguere il siero di uomo da quello di scimmia. Egli studiò pure le relazioni fra albume e tuorlo dell' uovo, e siero di pollo: e vide che, sensibilizzando con ciascuna di queste sostanze, si ottenevano reazioni

anche con le altre. Riuscì invece a distinguere i muscoli di pesce (trota) dalle uova o dagli spermi degli stessi animali. Ripetuto un esperimento analogo con le uova e con i muscoli di rana, anche in questo caso la distinzione riuscì perfettamente. Le cavie sensibilizzate con uova di rana invece reagirono, sia pure debolmente, alla reiniezione con estratto di girini; dimostrando così una graduale modificazione delle proteine durante lo sviluppo; e rispondendo perciò *ante litteram* al problema generale che doveva esser più tardi posto dal Kritchewsky (1914) in un interessante lavoro, basato sulla possibilità di constatare differenze biochimiche fra individui della stessa specie a diverso stadio di sviluppo e somiglianze biochimiche fra individui di specie diverse, uno stadio di una delle quali ripetesse o si avvicinasse a uno stadio dell' altra. Allo stesso problema rispondono anche le ricerche di Maunu af Heurlin (1911), il quale non riuscì ad ottenere risultati precisi sperimentando con albume e tuorlo d' uovo, e siero di pollo: mentre poté constatare alcune reazioni di affinità tra i sieri di pollo, di piccione e di anatra.

Negli anni successivi molti sono gli autori che hanno utilizzato l' esperimento dell' anafilassi a scopo di indagine biologica.

Yamanouchi (1910) riuscì a distinguere, nell' esperimento dell' anafilassi nella cavia, i sieri di uomo e di cimpanzé da quello di Macacus rhesus; non riuscì invece a distinguere fra loro i primi due sieri (questo autore non praticò tuttavia ricerche quantitative). Egli vide inoltre che in cavie sensibilizzate con siero di uomo o di cimpanzé, il siero di macaco, che non era capace di provocare lo choc, determinava però uno stato di antianafilassi. La differenziazione fra i sieri di uomo e di scimmia riuscì invece ad Uhlenhuth e Haendel (1910).

Il Graetz (1910) non riuscì, per mezzo dell' esperimento dell' anafilassi attiva, a scoprir differenze fra i sieri di varie specie di topi e ratti; ed attribuì questo insuccesso alla eccessiva delicatezza della reazione. Più recentemente invece, Otto e Cronheim (1925), con ricerche quantitative, riuscirono a differenziare i sieri di ratto e di topo nella cavia sensibilizzata attivamente e passivamente.

Rhein (1913) riuscì a distinguere le orine di specie diverse salvo che queste fossero molto vicine.

Glock (1914) riuscì con l' esperimento dell' anafilassi nella cavia a distinguere i sieri di due razze di galline che non avevano mostrato alcuna differenza alla prova della precipitazione ed a quella dell' immunizzazione crociata. Shirakawa (1925) riuscì a dimostrare delle differenze fra i sieri di asino, cavallo e mulo: mentre Sollazzo (1926), come già Uhlenhuth e Haendel, non riuscì a dimostrare differenze fra i sieri di cavallo ed asino.

Schwarzmann (1926) ha visto che l' esperimento della anafilassi passiva provocata nella cavia con siero di coniglio, impiegato per la distinzione tra specie vicine, dà risultati talvolta superiori e talvolta inferiori a quelli ottenuti con la precipitazione.

In alcune ricerche inedite compiute col Federici nel 1924 e troncate dalla immatura perdita di questo, avevamo ripreso in esame anche questo punto, ed eravamo riusciti ad anafilattizzare il coniglio con siero di lepre: mentre non riuscirono costanti i risultati ottenuti nella cavia.

In altri gruppi di organismi viventi, Kraus e Doerr (1909), Holobuth (1909), Kraus e Admirazibi (1910) hanno utilizzato con successo l' esperimento anafilattico per la differenziazione di specie batteriche vicine; mentre il Delanoë (1909) ottenne reazioni (sebbene con dosi più elevate) anche con specie batteriche assai diverse (*b. tifico* e *b. tubercolare*).

Karasawa (1910), Wendelstadt e Fellmer (1911), Fellmer (1914) utilizzarono l' anafilassi per la distinzione fra diverse albumine vegetali. Osborne e Wells (1911, 1913), usando proteine di origine vegetale, assai purificate e cristallizzate, riuscirono, p.e., a distinguere nell' esperimento dell' anafilassi le gliadine estratte dall' orzo e dal frumento, che pure, all' analisi chimica, presentano gli stessi aminoacidi, in proporzioni apparentemente uguali.

Altri autori hanno impiegato l' anafilassi per dimostrare la specificità o meno di diversi secreti rispetto all' organismo produttore. Così Minet (1912) poté dimostrare che l' orina sensibilizza anche per il siero della stessa specie: e Duhot (1913) poté dimostrare lo stesso per il liquor: ed il Seitz (1913) per la saliva.

Queste ricerche non rappresentano in fondo che un aspetto di quelle, numerosissime, che negli anni immediatamente precedenti la guerra furono eseguite, specialmente sotto la ispirazione delle idee dell' Abderhalden, per dimostrare l' esistenza di una specificità di organo oltre che di specie; concetto che, nella sua forma più rigida, non sembra potersi applicare che a poche formazioni (cristallino), ma che fu pure fecondo di risultati.

Recentemente il Biberstein (Biberstein, 1924; Biberstein e Lubinski, 1924) ha ripreso nell' uomo, per mezzo delle reazioni cutanee, lo studio della specificità di organo e di specie, giungendo a conclusioni interessanti sullo sviluppo nel tempo di ambedue e sulla graduale sostituzione della seconda alla prima.

L' esperimento dell' anafilassi può rivelare differenze assai delicate. Il Plotz (1924) ha constatato una netta differenza di specificità fra il siero vecchio ed il siero fresco: differenza che scompare facendo gorgogliare CO_2 attraverso il siero vecchio: onde questo autore conclude che la specificità non dipende soltanto dalla natura chimica del siero ma anche dal suo stato fisico-chimico. La stessa conclusione si può trarre dai risultati di Falk e Caulfield (1923), che lavorarono con proteine a diverso Ph.

Questi risultati ci conducono a parlare delle interessantissime ricerche di Dale e Hartley (1916), ampliate successivamente e fino a questi ultimi anni dal Doerr e Berger (1921, 1922) e dallo Stern (1922); i quali, in esperimenti quantitativi, sono riusciti a distinguere le diverse proteine del siero: ed a dimostrare come esista per queste, oltre che una specificità di specie, anche una di frazione egualmente sviluppata e basata probabilmente, come la prima, sulla struttura chimica delle proteine. Wells e Osborne (1921), lavorando con le proteine del latte, ottennero risultati analoghi.

Un progresso ulteriore in questo senso é rappresentato dalle ricerche di Dakin e Dale (1920), i quali, lavorando con le proteine purificate dell' uovo di gallina e di anitra, che contengono gli stessi aminoacidi in identiche proporzioni e che hanno identiche proprietà fisiche, riuscirono a dimostrare contemporaneamente una netta

specificità nell' esperimento anafilattico ed una certa differenza nei risultati della racemizzazione : indice quest' ultima, secondo il Dakin, di una differenza di posizione degli aminoacidi.

In queste ricerche abbiamo un primo esempio, coronato da successo, di applicazione dell' esperimento della anafilassi a delicate indagini biochimiche. A questo proposito é opportuno ricordare anche le interessanti esperienze del Quagliariello (1926), rivolte a dimostrare con l' esperimento dell' anafilassi eventuali diversità fra l' emocianina dei crostacei e quella dei molluschi.

A questo proposito é interessante leggere il libro del Wells (1924).

Le possibilità metodologiche dell' anafilassi non sono certamente esaurite con le ricerche sopracitate. Si può anzi dire che il campo sia stato appena sfiorato e che esso promette notevoli risultati a chi voglia intraprenderne lo studio sistematico. In particolare é da deplorare che il metodo non abbia trovato più larga applicazione presso i zoologi, come un valido criterio ausiliare, insieme agli altri fenomeni immunitari, per la distinzione o il ravvicinamento di specie. Anche in questo caso, come in troppi altri, i diversi rami della ricerca sono restati separati, come in compartimenti stagni ; e, se é a deplorare l' abuso delle applicazioni dell' anafilassi che si é spesso fatto in clinica, non é meno lamentevole che un fenomeno di tanta importanza abbia destato così scarsa eco nel campo dei naturalisti. Se questa rassegna avrà servito a destare l' interesse di qualcuno di questi ultimi e ad invogliarlo a un' indagine più profonda, se invece contribuirà ad infrenare gli entusiasmi di qualche clinico, esso avrà assolto al compito assegnatole.

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SALIENT FEATURES OF THE PROBLEM OF BUD-SCALE MORPHOLOGY

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CONTENTS.

1. INTRODUCTION.

Few of the specialised problems of plant morphology illustrate the effects of the general botanical progress of the past century and a half more clearly than that dealing with the bud scale. The explanation lies in the fact that the foliar nature of bud scales was recognised as early as 1682 by Grew and so the scale, as a type of "leaf," has in turn been interpreted from the standpoints of "idealistic" and "formal" morphology, as well as from the more recent evidence furnished by ontogeny and experiment.

In spite of its long and involved history, the developmental aspects of this problem, which were pointed out over fifty years ago by Mikosch, and firmly established by Goebel's classical work of 1880, have been greatly neglected in favour of a more formal or descriptive treatment. The static viewpoint of the problem, which appears all too commonly to-day in our general botanical manuals and texts, has done much to discourage active investigation. Indeed, one usually finds the bud scale dismissed with a brief account of its specific homologies, structure and function.

However, it has become increasingly evident, especially from the recent excellent studies on bud periodicity carried out in the laboratories at Wageningen, Holland[1], that a clear analysis of the factors regulating the alternation of bud scales and foliage leaves in woody plants is urgently needed, preparatory to a more thorough understanding of differentiation in the winter bud. Furthermore, the causes for the extreme divergence in the adult form and structure between the bud scale and the foliage leaf are obviously of fundamental importance, but are quite obscure at present.

During the past four years, the writer has been engaged in a complete re-examination of the problem of bud-scale morphology and in the course of this work a thorough study has been made of the historical aspects of the question. In the present paper, an attempt is made to discuss critically the salient features of this complex, and often inaccessible, literature, in the hope of clearly establishing the present status of the problem and of indicating the advances in our viewpoint which may be expected when more complete developmental and experimental data are available.

2. THE DISTRIBUTION OF BUD SCALES IN EXTINCT AND MODERN FLORAS.

Before proceeding to the more technical aspects of our problem, it will be well to note the widespread occurrence of cataphylls in vascular plants.

Although considerable light may be thrown on the time of origin of bud scales by future paleobotanical discoveries, the information at present from this source appears inconclusive. Thus, for example, Seward (1917, p. 221) says of *Cordaites*: "There is no proof that young vegetative branches with their spirally rolled leaves were protected by bud-scales, but some oval triangular scales, occasionally found in association with larger foliage-leaves, may have served that purpose." Whilst the "scale-habit" is undoubtedly very ancient, the occurrence of scaly buds among extinct Angiosperms is a problem which, in spite of its great theoretical interest, must remain for the present unsolved.

Amongst living Pteridophytes, cataphylls have been noted in *Marsilia hirsuta* A. Br. by Glück (1922); in *Selaginella, Isoetes, Osmunda* and *Onoclea* by Goebel (1880; 1884, p. 248; 1905, pp. 350–351)[2]; and in the two latter genera and in *Todea barbara* (L.) Moore by Bower (1884, p. 578).

Cataphylls are usually associated with the buds of underground rhizomes in Angiosperms and their presence has been recorded in many Monocotyledons and Dicotyledons by Goebel (1880) and Thomas (1900 *a*, 1900 *b*).

The great majority of the investigations on bud-scale morphology, however, have been based on the study of northern ligneous plants where the typical winter bud, with its outer envelope of scales, and its considerable differentiation of leaf and flower initials, apparently predominates[3]. Bud scales are of frequent occurrence

[1] Cf. the papers of Blaauw (1920), Versluys (1921), Luyten (1921), Luyten and Versluys (1921), Blaauw (1923), Bijhouwer (1924), and Luyten and De Vries (1926), cited in the bibliography.

[2] Cf. especially Goebel (1918, p. 1027 and pp. 1053–1054).

[3] Cf. the papers of Askenasy (1877), Albert (1894), Wiegand (1906), Moore and Behney (1908), and Moore (1909).

in the Gymnosperms and have been described in many genera of the Coniferales by Henry (1837, pp. 88–114), Goebel (1880) and Grüss (1885, 1892), in some of the Cycads by Goebel (1880) and Bower (1884); and in *Ginkgo biloba* L. by Goebel (1880) and Sprecher (1907, p. 53). It is amongst woody Dicotyledons, however, that scaly buds seem to reach their greatest development. In a relatively small number of species, *e.g. Viburnum Lantana* L., *Rhamnus Frangula* L., *Calycanthus floridus* L., *Robinia pseudacacia* L., etc., the buds are scaleless, but here protection is offered in a number of interesting ways which have been carefully investigated by Feist (1887). Save for these exceptional cases, scaly buds are known to occur in most of the northern Dicotyledonous families, including representatives of both the Archichlamydeae and Metachlamydeae[1].

Whilst the investigations of Treub (1887), Potter (1891), Groom (1892) and Dove (1896) have clearly shown that buds in the tropics are by no means devoid of protection, less definite information appears to have accumulated regarding the occurrence of bud scales in these regions. For example, Schimper (1903, p. 329) states: "The type of winter-bud with its large dry covering of scales and considerable differentiation, is foreign to the constantly humid rain-forest, whereas it reappears in dry forest and savannah." Schimper (*op. cit.* p. 349) found that in xerophilous trees of the tropics "the foliage-buds are provided with a coating of protective scales as thick as, or even thicker than, that of trees of the temperate zones"; the scaly buds of *Myrcia longipes* (Berg.), *Eugenia Jaboticaba* and *Eugenia dysenterica*, are cited as examples. Warming (1909, p. 115) endorsed Schimper's views regarding the absence of scaly buds in the tropical rain-forest. Other investigators, however, have found that scaly buds are not at all infrequent under moist tropical conditions. Thus Huber (1898) reported definite cataphylls in *Hevea braziliensis* Müll.-Arg., *Mangifera indica* L., *Mammea americana* L. and *Licania macrophylla*. The investigations of Holtermann (1907, pp. 183–186) on the vegetation of the tropical rain-forests of Ceylon show that in several Lauraceous genera, such as *Litsea* and *Actinodaphne*, large resting buds with well-developed scales are formed. Volkens (1912, p. 88) found that many trees observed by him in the gardens at Buitenzorg, Java, exhibited a definite rhythmical shoot growth involving the production of several "leaf broods" (Blattschübe) annually. The individual leaf broods, according to Volkens (*op. cit.* p. 87) are separated in many cases by bud scale scars, as in *Gustavia angusta* L., *Eriobotrya japonica* Lindl., *Barringtonia serrata* Mig. and *B. rubra*, *Litsea latifolia*, *Magnolia pterocarpa*, *Shorea pinanga* Scheff., *Ochrocarpus congregatus*, and *Alsodeia bengalensis*; or by the persistent cataphylls themselves as in *Actinodaphne procera* and *Daphniphyllum bancanum* Sulp. Kurz. Volkens further recorded bud scales in *Amherstia nobilis* Wall. and *Theobroma Cacao* L. Resvoll (1925) has approached the problem of bud covering in the tropics from a promising angle by investigating the behaviour

[1] Cf. Schumann (1889) and Brick (1914, pp. 216–217) for tabulated lists of plants with scaly buds. Further information is given by Willkomm (1864), Hitchcock (1893), Ward (1904), Schneider (1903), Lubbock (1897, 1908) and Trelease (1918). According to Glück's (1906) extensive investigations, cataphylls of various morphological types are also a prominent and characteristic feature of the highly specialised "turions" or winter buds of many aquatic Angiosperms.

of the cosmopolitan genus *Quercus* in Java. She found that the bud structure and scale anatomy of many of the tropical evergreen oaks are fundamentally similar to the conditions in European species.

In the present paper, the term "cataphylls" will be used synonymously with "bud scales" to designate the scale-like foliar organs which invest the resting bud. According to Jackson (1916, p. 67) the term "cataphyll" applies to "the early leaf-forms of a plant or shoot, as cotyledons, bud-scales, rhizome-scales, etc." It must be emphasised, however, that no rigid and inclusive definition of a cataphyll, based on its time of origin or position, is possible at present[1]. Many foliar organs, which are not limited in occurrence to subterranean or aerial resting buds, possess some of the salient external and internal features of "cataphylls," *e.g.* the reduced scale-like leaves of fleshy parasites, many bracts and prophylls (cf. Schmidt, 1889, p. 20 footnote 1), the primary leaves of *Vicia Faba*, etc. (cf. Goebel, 1900, p. 156). Under such circumstances, "cataphylls" is merely a convenient term for designating foliar organs protective in nature and usually less segmented than the foliage leaf.

3. THE IDEALISTIC "DOCTRINE OF METAMORPHOSIS."

Although Čelakovský (1885, p. 152, footnote 1) has attempted to show that Linnaeus used the term "folium" in a concrete sense to designate the foliage leaf, during the early phases in the development of morphology the diverse foliar organs of plants were simply classified as types of "leaves" and no attempt was made to indicate their relationship to each other by means of a generalised theory. Thus, Grew (1682, p. 32) described bud scales and stipules as the protective "surfoyls" and "interfoyls" of the bud; and Malpighi (1686, p. 22), who discussed and illustrated the "transitional" cataphyllary series in many species, referred to the lower appendages of the bud as "manifold classes of leaves, with the shape of scales."

As the knowledge of plant organography increased, however, the need for a broad concept of the "leaf" was felt and there appeared the well-known "Doctrine of Metamorphosis," which, it was hoped, would provide "the key to morphology."

Under the idealistic "Doctrine of Metamorphosis," the bud scale stood in no *genetic* relationship to the foliage leaf, but was considered to represent *one* of the visible expressions of the "abstract idea" conveyed in the word "leaf." Goethe, for example, attempted to show[2] that whilst the outer "leaves" of the winter bud appear as scale-like structures, the following cataphylls become progressively narrower and more segmented apically until finally the previously scaly organ has "contracted" to the petiole of the foliage leaf. Later writers such as Nees von Esenbeck (1820, pp. 476–477) and Regel (1843) regarded bud scales as "leaves" which have remained at the lowest stage of leaf metamorphosis, which they held was represented by the "stipule formation." This idea finds some correspondence in the theories of Agardh (1849) and Clos (1879), who attempted to prove that stipules are organs *sui generis*.

A further result of the idealistic viewpoint of metamorphosis appeared in the form of several generalised characterisations of scales. According to Goebel (1905,

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[1] Diels (1906) has shown that flowering is not limited to any specific age in plants.
[2] Cf. Goethe's *Werke*, 1891, p. 329.

p. 384), Schimper (1830) originally applied the expression "Niederblätter[1]" to the leaves of subterranean shoots. In his later paper, Schimper (1835, p. 43) attempted to show that in many perennial herbs and in woody plants the foliage leaves are preceded by a sheath formation ("Scheidenbildung") which is primarily characterised by the absence of a lamina and is represented by bud scales and the scale-like leaves of hypogeous shoots and fleshy parasites. In contrast, according to Schimper (*op. cit.* p. 44) the lamina is predominantly characteristic of the "foliage-leaf formation."

Schimper's ideas were further elaborated by Braun (1851) whose definition of the "cataphyllary formation" is typical of the idealistic viewpoint. He writes (*op. cit.* p. 66) that cataphylls "are remarkable from their broad basis, small height, and most simple shape and nervation[2]; they have no laminae, no stalks, no sub-division[3], consequently never have stipules, and are constantly entire."

Rossmann (1857, pp. 31–47), however, maintained that Braun's definition of the "Niederblattformation" was (1) incorrect in that it postulated the absence of petioles, and (2) that it possessed no general applicability, since many of its supposed characteristics were also shown in other foliar organs, such as bracts, sepals, etc. Rossmann concluded on the basis of "transitional" forms and venation that cataphylls (*i.e.* bud scales and the scale-shaped leaves of subterranean shoots) are either "true" phyllodes or petioles with adnate stipules and that the lamina takes no part in scale construction but appears as a "new" structure in the intermediate forms.

That the abstractions involved in the idealistic morphology of Goethe, Schimper, Braun and Rossmann have only obscured the path leading to the real meaning of the diverse "leaf-forms" in the flowering plant is generally admitted to-day[4]. The "lower sheath" or "cataphyllary formation," as defined by Schimper and Braun has proved a highly artificial conception, and is an attempt to delimit foliar organs which are frequently morphologically identical with hypsophylls (cf. Goebel, 1884, pp. 250–251 and 1905, p. 390, and Glück, 1919, pp. 165–166). Unfortunately, the idealistic view of metamorphosis has not been entirely abandoned as the result of later ontogenetic and experimental investigations, but, as will be shown later, has appeared under the guise of several phylogenetic theories and as the basis of what Goebel has termed the "Differentiation Theories."

4. THE "FORMAL" OR DESCRIPTIVE VIEWPOINT.

Considerable attention has been devoted in the past to a study of the specific homologies of bud scales and an extensive literature has accumulated regarding the relative importance of the sheath, leaf base, stipules, petiole and lamina in scale construction.

[1] This term, which is very commonly used in the German literature to designate bud scales, was originally rendered in English as "cataphyll" by Henfrey (1853, p. 4, footnote).

[2] *I.e.* usually the parallel type.

[3] Except the stipular scales of some Amentiferae, which Braun considered a "transition" to the "foliage-leaf formation."

[4] For a complete discussion of the history and significance of the "Doctrine of Metamorphosis," reference should be made to Wigand (1846), Lewes (1875, pp. 352–354), Goebel (1880, 1884, 1895 *a*, 1895 *b*), Čelakovský (1885), Sachs (1890), Green (1909) and (Haecker) (1927). The most comprehensive account is given by A. Hansen in his "*Goethes Metamorphose der Pflanzen*," Giessen, 1907.

An interesting feature of some of the earlier investigations along these lines is the attention which was devoted to classifying buds according to the morphology of their scales. From this standpoint, *the bud covering as a whole*, rather than the scale as a type of "leaf," constituted the unit for investigation.

Probably the earliest attempt at a precise classification of buds is found in Löfling's (1751) *Gemmae Arborum* which was written to establish a "system" of buds according to their structure (*op. cit.* pp. 196 *et seq.*). Löfling (*op. cit.* p. 185) distinguished the "embryo of the future plant" (*i.e.* the rudimentary leafy shoot) from its scaly envelope which he termed the "bud" or "gemma." As the result of this artificial distinction he stated (*op. cit.* p. 188) that a number of temperate and most tropical trees "lack" buds, since their young shoots are not protected by scales, a viewpoint later developed by De Candolle (1841). Löfling (*op. cit.* p. 185) conceived of the inner scales of opening buds as elongating and progressively "expanding" more and more into the "perfect" leaves. He found that the manner in which this may be achieved varies according to the species and on this basis he recognised "buds" composed of "folia" (*gemmae foliaceae*), of petioles (*gemmae petiolares*), of petioles with adnate stipules (*gemmae stipulaceo-petiolares*) and of stipules (*gemmae stipulaceae*). In Löfling's remarkable treatise, a classification of the buds of 108 species of woody plants is given based upon the morphology and phyllotaxis of their scales. The number of scales and the vernation of the leaves within the "bud" furnished characters for grouping the species into "orders." Löfling's classification of buds was incorporated in Linnaeus' (1790) *Philosophia Botanica* and adopted by Leendertz (1832) in his speculative essay on the bud.

A number of subsequent writers shared Löfling's viewpoint regarding the classificatory value of the "bud." Mirbel (1815, pt I, p. 139), for example, applied the term "perula[1]" to the outer envelope of buds which he maintained is formed either of abortive leaves, petiole bases or stipules. Mirbel (*op. cit.* pt II, p. 635) also noted that a "perula" is absent in some plants and referred to the bud in such cases as "nuda[2]."

De Candolle (1841) believed that bud scales were the result of "the semi-abortion or degeneration of the foliaceous parts," and expressed the opinion that the transitional forms appearing in opening buds of the ash or sycamore (*Acer pseudoplatanus* L.) make it "impossible to doubt that the external pieces are of the same nature as the internal." In spite of this relatively advanced idea, he adopted Löfling's view and designated the collection of scales or coats investing the young shoot as the "bourgeon." He classified (*op. cit.* pp. 285–286) "buds" as *foliaceous, petiolaceous, stipulaceous* and *fulcraceous*. This latter class is synonymous in meaning with Löfling's "stipulaceo-petiolares" and was adopted as late as 1885 by Duchartre. De Candolle's classification of buds was later taken over by Broers (1833) and

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[1] The expression "perula" has persisted in literature being adopted, for example, by Duchartre (1885), Godfrin (1894), Van Tieghem (1898) and Bugnon (1926 *b*). Schneider (1913) has termed the persistent or deciduous collection of scales at the base of the spur shoots in *Pinus* a "Niederblatt-scheide."

[2] Scaleless buds are usually described in modern texts as "naked," a singularly inappropriate term since "protection" is achieved in many other ways. Cf. Feist (1887) for details. Brick (1914, p. 219) has applied the terms "open" and "closed" respectively to the scaleless and scaly type of bud.

formed the basis of C. De Candolle's (1862) synoptical treatment of the bud types in the Juglandaceae.

A second attempt at an extensive classification of buds, primarily based on scale morphology, is found in Áime Henry's (1846) *Knospenbilder*. In this unique work the buds of about 129 species of plants (both Gymnosperms and Angiosperms) are described and illustrated from the standpoints of the arrangement and morphology of their scales and the vernation of their leaves. Although Henry's studies clearly show the strong influence of the prevailing theories of idealistic morphology and spiral phyllotaxis (cf. Bravais (1837)), his analytical descriptions and diagrams of bud structure remain permanently valuable. Henry recognised (1) the "blatt-deckige Knospen," a class more or less identical to Löfling's "gemmae foliaceae," (2) the "blattstieldeckige Knospen," and (3) the "Nebenblattdeckige Knospen" under which he grouped all buds occurring on stipulate-leaved plants, regardless of the relative importance of the stipules in scale construction (cf. Clos (1879, pp. 192–193) for additional information regarding "bud" classification).

A number of the early workers, however, were less concerned with an attempt at "bud" classification and devoted more attention to the scale itself. One of the interesting results of this type of investigation lies in the doubt which arose as to the possible rôle of the petiole in scale construction, a doubt which increased with knowledge of leaf development. As early as 1824, Link, who applied the expression "tegmenta[1]" to the outer coverings of buds, noted (*op. cit.* p. 212) the abortion of both lamina and petiole and the predominance of the sheath (vagina) in cataphyll formation. Schleiden (1843) objected to the expression "perula" and applied Link's term "tegmenta" to the cataphylls of both aerial and subterranean buds. He (*op. cit.* p. 203) recognised *tegmenta foliacea*, *tegmenta stipulacea* (in place of Link's "ramenta"—cf. also Rossmann, 1857, p. 43, footnote 1) and *tegmenta vaginalia* (*i.e.* the bulb scales of *Allium*). Clos (1856), however, appears to have been the first to emphasise the importance of the leaf sheath in the interpretation of bracts, sepals and bud scales. Many previous investigators had recognised but two "formal" elements in the foliage leaf, *i.e.* the petiole and the lamina. Clos on the contrary, found that the bracts and sepals of a number of species represent "un grand développement de la gaîne avec atrophie concomitante du limbe[2]." He concluded from this that the sheath is always present in leaves at least virtually. In applying this theory to the interpretation of bud scales, he maintained (*op. cit.* p. 684) that in *Aesculus hippocastanum* L. whose "buds" had been classed as "petiolaceous" by De Candolle (1841, pp. 285–286), the cataphylls are really derived from the sheath. For such cases he proposed the term "bourgeons vaginaux," but nevertheless did not completely reject the "petiolaceous bud" as a possible type in other plants. Clos further modified De Candolle's classification by suggesting the adoption of the more specific expression "bourgeons limbaires" in place of the indefinite "bourgeons foliaces." In a later paper Clos (1879, pp. 192–

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[1] This term has been widely adopted by German morphologists, *e.g.* Mikosch (1876), Wiesner (1884) and Neese (1916).

[2] An essentially similar viewpoint was reached by Goebel (1884) and Schmidt (1889) on onto-genetic evidence.

193) applied the term "vagino-stipulaires" to the bud scales of *Oxalis*, *Mahonia* and many of the Rosaceae.

Many of the later interpretations of bud scales were probably influenced by the ontogenetic studies of Mikosch (1876) and Goebel (1880), and, with few exceptions (cf. Henslow, 1901, p. 27) either rejected the petiolar scale in favour of the vaginal or leaf-base type, as was done by Wiesner (1884, p. 46), Vines (1895, p. 59), Van Tieghem and Costantin (1918, p. 308) and Strasburger (1921, p. 176); or at least included the vaginal scale as an additional type. Duchartre (1885, p. 536), for example, emphasised that only the *bases* of the petioles are modified into scales in the so-called "petiolaceous bud." Lubbock (1908, p. 105) who recognised four "elements" in a "complete" leaf, on the other hand did not distinguish the leaf base in exstipulate plants such as *Acer* and *Fraxinus*, and interpreted the scales here as modified leaf stalks.

The question of the true nature of the so-called prophylls[1] of winter buds represents another problem which was approached very early from the "formal" standpoint. Turpin (1819, p. 15) seems to have been the first to point out that the first scale or leaf on Monocotyledonous shoots is addorsed, whilst the axillary bud in Dicotyledons usually begins with two opposite lateral scales or more rarely with an anterior scale addorsed to the subtending leaf. He interpreted (*op. cit.* p. 15, footnote) the addorsed position of the prophyll in Monocotyledons, and, when it occurs, in Dicotyledons, as the result of the fusion of two opposite lateral prophylls which he held was the "original" condition. Several later investigators, such as Henry (1836–1846)[2], Döll (1848), and Eichler (1875–1878) used this supposedly invariable prophyllary distinction between Monocotyledons and Dicotyledons as an aid in the interpretation of the outermost scales of the winter bud. A deviation from the "typical" position of the prophylls, *e.g.* an abaxially or adaxially addorsed insertion of the first scale of the bud, was regarded as a case of "fusion" in many instances.

The descriptive phase of the problem must now be left; and a more critical judgment of the various types of bud scales reserved until the ontogenetic aspects of the question have been taken up.

5. THE NATURE OF THE BUD SCALE IN THE LIGHT OF DEVELOPMENT, EXPERIMENT AND ANATOMY.

(i) *The Influence of Studies on Leaf-development.*

The characteristic feature of both the "idealistic" and "formal" interpretation of the bud scale is the absence of ontogenetic evidence, *i.e.* the burden of proof is largely placed on phyllotaxis and the seriation of leaf forms on the axis. The morphology of the scale, however, appeared in a new and dynamic light when its history of development was compared with that of the foliage leaf. Probably the most important contribution of the earlier work on leaf development to our problem

[1] Wydler (1843, p. 154) originally applied the term "Vorblätter" to the first two members of the leafy shoot.
[2] Henry (1846, p. 212) applied the term "Knospenkeimschuppen" to the prophylls of winter buds, probably in allusion to their analogy with the paired cotyledons of Dicotyledons.

lies in its analysis of the primary segmentation of the leaf, i.e. the ontogeny of its "formal" elements.

Trécul (1853) pointed out that many leaves consist of three elements: the sheath, the blade and the petiole, and that these divisions of the leaf usually appear in the order given above. He found that in a large number of cases the *sheath may exist alone* which led him to emphasise (p. 244) that in all Monocotyledons and Dicotyledons with sheathing leaves, the sheath always arises first in ontogeny.

Eichler (1861), in contrast to Trécul, found that the first recognisable leafy structure at the growing point is an annular or crescentic primordium which he termed (p. 7) the "Primordialblatt." According to Eichler (pp. 7–8) in all Phanerogams this "Primordialblatt" first segments into a stationary leaf base (the "Blattgrund" or "Unterblatt"), and an upper leaf ("Oberblatt"). The leaf base either takes no further part in leaf development or else forms stipular appendages; the sheath, when present, was held by Eichler to arise *secondarily* by intercalary growth from the leaf base. The upper leaf gives rise to the petiole and the simple or segmented lamina. Like Trécul, Eichler emphasised (p. 9) that the petiole arises *last* in ontogeny, but further maintained (p. 8) that it is not directly inserted upon the axis, but rather upon the leaf base. The petiole itself, according to Eichler, develops from a short meristematic zone interpolated between the leaf base and the upper leaf. In the case of compound or lobed leaves, and in many simple leaves, he found that the petiole only differentiates after all the segments of the mature lamina have begun development. In some instances, however, as in *Liriodendron*, the petiole arises during, or previous to, the formation of the individual lobes of the mature lamina, but in no case did he find the petiole developing prior to lamina differentiation.

The fundamental researches of Trécul and Eichler formed the real basis for subsequent ontogenetic studies on bud scales which can now be discussed.

(ii) *The Work of Mikosch.*

Although it is evident that Clos' (1856) recognition of a vaginal type of scale was influenced by the work of Trécul (1853), Mikosch (1876) appears to have been the first to adopt Trécul's results in an ontogenetic study of the bud scale.

Mikosch recognised two general types of bud-coverings or "tegmenta." In one type, the bud is protected by the persistent base of the axillant leaf which Mikosch (p. 752) termed the "Articulartegment." Although the persistent base of the subtending leaf cannot be strictly classed as a bud scale (since it is *not* an appendage of the bud-axis except in terminal buds), the "Articulartegment" was later recognised by Wiesner (1884, p. 46) and Feist (1887, p. 339); and its anatomical structure in *Smilax hispida* was described by Brick (1914, p. 295).

In the second type of "covering" Mikosch (pp. 752–753) stated that the bud scales arise from unequally differentiating leaf primordia in which only the vaginal, laminar or stipular portion of the foliage leaf clearly develops, whilst the other leaf elements either fail to grow or else soon abort.

Mikosch found that in a number of species of *Acer*, the bud scales consist of an

early differentiated basal portion, homologous to the sheath[1] of the foliage leaf, and a rudimentary apical lamina; the petiole, except in the innermost bud scales, is not developed[2]. He termed (p. 743) such scales, which he also found in *Aesculus*, *Fraxinus*, *Sambucus* and *Mahonia*, "Vaginaltegmente."

In other plants, such as *Cornus* and *Lonicera caprifolium* L. Mikosch (pp. 744–745) found that whilst the outermost pair of scales segment into petiole and blade[3], the bud is only covered by the laminar portion of these organs; he named such cataphylls "Laminartegmente."

Mikosch (*op. cit.* pp. 745–750) also investigated the ontogeny of the bud scales in a number of stipulate-leaved species. In the case of *Tilia grandifolia* Sm., he found that the foliage leaf early segments into two rapidly growing lateral portions, *i.e.* the stipules and a slowly developed median portion, *i.e.* the blade. The bud scale of this species, on the other hand, exhibits no segmentation but develops throughout as a single structure which Mikosch interpreted as representing two stipules "fused" with their leaf. The bud scales of *Platanus* were found to be of the same general type except that the free margins of the scales fuse during ontogeny to form cap-like structures. In *Quercus pedunculata* Ehrh., Mikosch found that whilst the outer scales are similar to those in *Tilia*, the inner ones represent pairs of modified stipules. Cataphylls of the type which have been mentioned were called "Stipulartegmente" by Mikosch.

A general criticism of Mikosch's work must be reserved until later. For the present it may serve to illustrate two important facts: (1) that ontogeny appears to lend no support to the so-called petiolaceous scale, and (2) that whilst bud scales seem to arise from foliage-leaf primordia in some cases, their ontogeny in other instances may be completely different from that of the foliage leaf (*e.g. Tilia*).

(iii) *Goebel's Interpretation of the Bud Scale.*

Goebel, in his well-known *Beiträge zur Morphologie und Physiologie des Blattes* (1880) and in numerous subsequent publications (cf. the papers cited in the bibliography) has brought forward considerable evidence to show that bud scales stand in a direct *genetic relationship* to the foliage leaves on the shoot. In contrast to the idealistic conception of metamorphosis advanced by Goethe, Schimper and Braun, Goebel maintains that the formation of a bud scale involves the *real metamorphosis*[4] of a foliage-leaf primordium. This idea is clearly set forth in his earliest paper (1880, p. 758). Thus, in Goebel's opinion, leaf metamorphosis is primarily an ontogenetic process in which the path of development of originally identical primordia, *i.e.* foliage-leaf primordia, is diverted into other channels as the result of internal and external changes. Goebel (1895 b, p. 212) writes: "All the primordia, for

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[1] Mikosch (p. 739) termed any lateral broadening of the leaf base a "sheath," and, like Trécul, regarded this element, when present, as appearing *first* in ontogeny.

[2] He also noted (p. 742) the drying-out and frequent abscission of the rudimentary lamina from the outer scales of this type.

[3] A sheath is not developed here according to Mikosch.

[4] Čelakovský (1885, p. 148) attempted to show that the idea of a "real" metamorphosis, which has been largely established by Goebel's classical work, had already been foreshadowed in the "Prolepsis" of Linnaeus.

example, of foliage leaves, cataphylls, and hypsophylls are identical, not because they are 'leaf primordia'—that is a useless abstraction—but because they are *foliage-leaf primordia*, structures having a definite material composition and developmental norm." Furthermore, according to Goebel, the phenomenon of metamorphosis is always related to a change in function, and is not limited merely to the transformation of organ primordia. As supporting evidence, he mentions the actual metamorphosis of the basal portions of the foliage leaves of *Lilium candidum* L. into storage organs, and writes (1900, p. 9): "The leaf has become *transformed*; it was first of all an organ of assimilation, later it has become in its lower part an organ of storage." Furthermore, "in some species of *Astragalus* and *Caragana*, the midribs of the pinnate leaves become thorns after the pinnules have fallen away." Goebel (*loc. cit.*) remarks: "Let us now assume, by way of example, that the leaf-pinnules in *Astragalus* fall away before they unfold, and before therefore they could act as assimilating organs, whilst the midrib develops into a thorn—would this not be a case of actual transformation? Of course it would; the change has only been advanced a stage[1]."

Mikosch (1876, p. 753) had already noted the frequent similarity in the early developmental stages of bud scales and foliage leaves as well as the strong divergence between these organs at maturity, but had not framed a general theory to account for this phenomenon. Goebel, however, attempted to characterise bud scales on the basis of their development, as "arrested formations" (Hemmungsbildungen) of foliage leaves. He (1884, p. 251) clearly defined his use of this expression as follows: "...cataphylls and hypsophylls on the one hand are *arrested formations* of foliage leaves, which do not originate simply because a foliage-leaf primordium stands still at a definite stage in development, but after this arrest there usually follows a further development of either the leaf base, the upper leaf or the primordial leaf, which diverges from the usual ontogeny. These two factors are to be kept well apart, on the one hand the identity with the foliage-leaf primordium up to a certain stage in development, and then from this point onwards the divergence of the development." Goebel has applied this theory, together with a revision[2] of Eichler's ideas regarding leaf segmentation, in an interpretation of the specific homologies of bud scales. He recognises four general developmental types, viz.:

1. In his early paper Goebel (1880) maintained that the cataphylls of such plants as *Syringa*, *Lonicera*, *Daphne*, etc., have arisen from foliage-leaf primordia arrested at a middle stage in ontogeny, and hence are equivalent to sessile leaf blades. This category embraced the "foliaceous" or laminar scales of the older investigators. More recently (1923, p. 1394) Goebel has included scales of this type, as well as the "double" scale of *Salix* under a group in which "the whole leaf primordium under more or less far-reaching changes, becomes a cataphyll."

2. In other plants, such as *Fraxinus* and *Acer*, Goebel found that the bud scale is formed from the enlarged and modified leaf base of the primordium; the lamina

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[1] As a matter of fact, the laminae of the outer bud scales in many species frequently drop off in a rudimentary condition. Cf. Mikosch (1876), Goebel (1880) and Versluys (1921, p. 167, Fig. 9).

[2] Goebel (1884, p. 215) has shown that the differentiation of the primordial leaf into leaf base and upper leaf is not always as distinct as Eichler maintained.

is laid down, but soon ceases growth and the intercalary petiole does not develop (cf. Goebel, 1900, pp. 6–7 and Fig. 1). Goebel (1880) pointed out that the so-called "petiolaceous" buds of De Candolle were based upon a failure to distinguish between the leaf base and the petiole. De Candolle's idea of the "fulcraceous" type of bud was also corrected by Goebel in the case of *Prunus padus* L. Here, the outer scales of the bud are of the leaf-base type with a rudimentary apical lamina and have arisen, according to Goebel, from the transformation of primordia whose leaf base had not yet laid down stipules. The inner scales, which are tridentate, have developed, in Goebel's opinion, after the foliage-leaf primordia had begun to form stipules. Thus, these cataphylls are also to be regarded as modified leaf bases, in which the arrested stipules and leaf blade have reached an apical position, and *not* as petioles with adnate stipules.

3. In a third series of plants (*e.g. Quercus*) the lamina is arrested in development and the bud scales are formed of the modified stipules. This category is similar to the "stipulaceous" buds of the early workers except that Goebel (1880, p. 774) emphasised the presence of microscopic laminae in connection with even the lower pairs of scales; only the "prophylls" of the bud are simple structures (cf. also Goebel, 1923, p. 1395).

4. Goebel (1884, p. 246) found that whilst the transformation of the foliage-leaf primordium to a scale occurs at a relatively late stage in Dicotyledons, the conditions are frequently simpler in Monocotyledons. The cataphylls of *Glyceria spectabilis*, for example, were found to arise from unsegmented "primordial leaves." Such cataphylls, as well as the numerous bracts which develop similarly (cf. Goebel, 1884, pp. 242–243 and 1923, pp. 1402 *et seq.*) are interpreted by Goebel as transformations of foliage-leaf primordia in which there has occurred an arrest of the lamina development, the absence of petiole formation and an increasing growth of the leaf base. Finally, a sheath-like organ develops in which no separation of the upper leaf from the leaf base is possible. Goebel (1900, p. 7) probably refers to such a divergent development of the scale when he says: "It may frequently happen that the transformation takes place much earlier and is then of course no longer capable of *direct* proof, but only to be concluded upon comparative grounds." (A further discussion of this point in regard to a number of doubtful cases will be taken up later.)

Goebel finds additional evidence of the genetic relationship between bud scales and foliage leaves in the well-known transitional or intermediate forms upon which previous morphological interpretation had so largely rested. He draws attention to the reduced nature of the lamina of the foliage leaves just preceding the outer scales of the terminal bud, and concludes that these transitional forms arise from foliage-leaf primordia arrested and modified somewhat later in ontogeny than occurs in the formation of the proper scales (cf. Goebel, 1900, p. 9).

Cogent experimental evidence has also been furnished by Goebel in support of his theory of the bud scale. He found that when young shoots are defoliated or "tipped," the axillary buds are induced to shoot out and then frequently produce foliage leaves, whose internodes elongate, instead of bud scales. A very significant

feature is illustrated by his experiments with *Prunus padus* where in many cases the *lowermost appendage* of the precociously expanding bud closely resembled one of the *innermost transitional forms* of a normally opening bud. Goebel found that these intermediate organs were experimentally called forth when the defoliation was performed later in the spring, *i.e.* after some differentiation had occurred in the bud. These mutilation experiments, which gave similar results with *Aesculus, Acer, Rosa, Syringa* and *Quercus*, strengthened the ontogenetic evidence and convincingly showed the identity (in these cases) of the primordia of the bud scale and foliage leaf. (For the details of these experiments cf. Goebel, 1880.) Goebel (1887) was also able, by means of defoliation, to cause lateral branching in *Onoclea struthiopteris* Hoffm. on which many graduations between normal fronds and sporophylls appeared. Goebel maintains that this plant only produces foliage-leaf primordia, a part of which are transformed to cataphylls and sporophylls, whilst the remainder develop further to foliage leaves. His idea of the inherent nature of these primordia is worthy of note (*op. cit.* p. lxxiv): "When here, as in other cases, mention is made of foliage-leaf primordia, not only is the chlorophyll content of these organs meant but also their entire individuality, which is characterised by a definite history of development as well as by their outer conformation."

Lastly, Goebel (1900, p. 195) supports his theory by an interesting teratological observation. He found that in several species of *Juncus* attacked by the insect *Livia juncorum* "the vagina (of the leaves) is greatly enlarged whilst the lamina remains small or may be entirely aborted, in other words, *the insect brings about here the same changes which take place otherwise in the normal 'leaf metamorphosis' when a cataphyll or a hypsophyll arises from the primordium of a foliage-leaf.*"

Goebel's investigations on bud-scale morphology, especially his ontogenetic and experimental studies, constitute the basis of modern research in this field, and his work has received direct corroboration from several investigators such as Mann (1894) and Glück (1919, pp. 165–166 and 1922, pp. 251–252). On the other hand, a number of objections have been raised against his theory of a real metamorphosis, partly as the result of a complete misconception of his viewpoint. These contrary views of "metamorphosis" may now be taken up.

(iv) *Other Developmental Views of "Metamorphosis."*

In the light of numerous investigations, the distinctions usually drawn between the stem and the leaf appear relative and not fundamental. Nevertheless, as many writers have pointed out, the "*Doctrine of Metamorphosis,*" as well as more recent theories of "leaf transformation," *necessarily* recognise the "leaf" as a morphological unit separate from and "inserted" upon the stem[1].

It has already been stated that the notion of the leaf was held in an abstract sense by such workers as Goethe, Schimper and Braun, who conceived of metamorphosis as a theoretical process. Wolff's (1759) "*Doctrine of Epigenesis,*" however, suggested to a number of investigators a more concrete method for studying the

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[1] This point cannot be pursued further here. Consult Bower (1884 and 1887), Goebel (1900, pp. 13–16) and Green (1909, pp. 65–84) for further details.

"transformations of the leaf." The viewpoint developed from this line of attack has given rise to what Goebel (1884, p. 108) termed the "Differentiation Theories."

Wigand (1846, pp. 5–6) maintained that the "ground form" underlying the manifold foliar organs of the stem is the "leaf primordium." He believed that whilst the history of development of the leaf primordia to various types of "leaves" could be regarded as a "real metamorphosis," one could only speak of the "transformation" of one organ into another in a figurative sense. Wigand considered that the progressive change in the "formative material" in the developing shoot was reflected in the seriation of leaf forms and that this alone is the real meaning of metamorphosis in plants.

Wigand's general viewpoint was shared by several later workers. Grüss (1892, p. 640), for example, homologised bud scales with leaves or their stipules, but believed that a primordium at the growing-point of the shoot may give rise to either a scale or a leaf. He writes: "Its development depends on outer influences, as light and heat or upon inner ones, such as food movement. All these factors determine which of these two organs is formed from the primordium." A precisely similar theory was advanced by Frank (1893, p. 3) who denied that leaves with a definite mature form ("von einer bestimmten Ausbildungsform") can actually be "transformed" into other organs, and held that the various leaf forms only have this in common—that they arise from embryonic, undifferentiated primordia. (The consequence of this viewpoint on the interpretation of the specific homologies of scales will be treated later.)

Goebel[1] has vigorously and ably opposed this viewpoint of metamorphosis which has resulted, to some extent at least, from a misconception of his own theory. He clearly stated (1895 b, p. 213) that by "real metamorphosis" is meant the transformation of *organ primordia*, not *mature* organs. According to Goebel, the "Differentiation Theory" represents a developmental interpretation of the idealistic "Doctrine of Metamorphosis" in that no *genetic relationship* between the bud scale and the foliage leaf is postulated—both organs arise from "indifferent leaf primordia" according to the prevalent external and internal factors. Goebel, however, takes the foliage-leaf primordium as the unit, which may become moulded and shaped during ontogeny to any of the various leafy members as the result of a functional change. He writes (1900, p. 6): "We speak of the scale-leaf as a transformation of the foliage-leaf, because in the juvenile condition we often find it to be possessed of parts which unfold themselves in the foliage-leaf, but become arrested in the scale-leaf; and further, we can experimentally hinder this transformation." In short, the frequent association of a rudimentary lamina with a bud scale convinces Goebel that the scale cannot have arisen from an "indifferent leaf primordium." Goebel's experimental work, which has already been outlined, also lends additional support to his theory. Further discussion of the "Differentiation Theory" must be postponed until the "causal" side of our problem is taken up.

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[1] Cf. Goebel (1884, pp. 108–110), (1895 b, pp. 211–215) and especially his *On metamorphosis in Plants* (1895 a).

Both the supporters of the "Differentiation Theory" as well as Goebel regard "metamorphosis" almost entirely as an *ontogenetic process*. The objection has been raised, however, that this viewpoint entirely neglects the evolutionary history of plant organs. Goebel's ideas, for example, have been severely criticised by Prantl (1884, pp. 52–56) who maintained that material influences may affect "leaf" development even before the appearance of the primordia themselves. The fact that during the "normal" undisturbed growth of the plant there is a constant production of different foliar organs led Prantl to conclude that the "leaf primordia" must be *different* from the very beginning. He thus regarded every primordium as the initial of the organ developing from it and so assumed the existence of distinct foliage-leaf primordia, cataphyll primordia, etc. Prantl, therefore, held that "metamorphosis" can only be considered phylogenetically and hence that Goebel is quite incorrect in deriving bud scales, for example, from foliage-leaf primordia— the scale has originated phylogenetically from the foliage leaf, not ontogenetically. (A similar view of metamorphosis was held by Reinke (1897, pp. 573–574) especially regarding the nature of the phyllodes in *Acacia*.) Worsdell (1915, p. 152) likewise supports what may be called the "Congenital Theory." He writes that "Goebel... cannot surely have any right to assume that foliage-leaf nature inheres in every rudiment, for the modification into bract, scale-leaf, etc., must in most cases be congenital."

Whether "evolutionary specialisation" has proceeded so far in some plants that the bud scales fail to show any trace of foliage-leaf nature even in their earliest developmental stages constitutes an open question at present and will be taken up again in another connection.

(v) *The Viewpoint of Anatomy*.

The inner structure of the bud scale has received considerable attention in the past. The work of a number of the investigators in this direction, such as Schacht (1859, pp. 97–98), Areschoug (1870), Cadura (1886) and Schultz (1888) was largely descriptive in nature, and little or no attempt was made to interpret developmentally the anatomical differences between scales and foliage leaves. Cataphylls were regarded by some observers as structurally "adapted" for their special protective functions because of the frequent appearance of "mechanical tissues" in them, such as collenchyma, sclerenchyma, periderm, heavily thickened epidermal walls, etc. (For further details cf. Droit (1908), Pantelievskij (1910), Wiegand (1906) and Grüss (1885, 1892).) A reduction in the number of stomates and the development of the vascular bundles was noted by other workers, and Adlerz (1881) in particular, found that in most bud scales, a differentiation of palisade and spongy parenchyma tissue does not occur.

The histological features of the bud scale, however, appeared more significant when their development was studied. Mikosch (1876, p. 753) found that the anatomical structure of the scale completely agrees in the first developmental stage with that possessed by the region of the leaf, with which the scale is homologous,

at a similar period. *Subsequently* according to Mikosch, the tissues experience such changes as adapt the scale to its particular physiological function. Wiesner (1885, p. 130) likewise held that bud scales show many anatomical similarities with the region of the foliage leaf with which they are equivalent. Schumann's (1889) monograph is based upon an investigation of the bud-scale anatomy of 135 species of Gymnosperms and Angiosperms, and treats the subject comparatively. Several of his observations are of particular interest here because of their relation to Goebel's interpretation of the scale. Schumann (*op. cit.* p. 9) found that palisade parenchyma is absent in bud scales, a fact which seemed of interest since many cataphylls supposedly arise from foliage-leaf primordia arrested at a middle stage in ontogeny. He further observed (*op. cit.* pp. 20–21) that the venation of a scale is dependent both upon its specific morphology as well as the degree of its "transformation." For example, in the case of leaf-base scales, the veins are unbranched and converge towards the apex in the outer bud scales, whilst a more complex branching system obtains in the inner ones. Schumann (*op. cit.* p. 25) concluded that bud scales (although they are foliar organs) are characterised by three negative anatomical features, i.e. the absence of assimilating tissue, stomates and a well-developed vascular system[1].

The papers which have just been mentioned seem to indicate that *some* of the anatomical features of the bud scale are related to its specific homology, and further suggest that the scale may experience to some extent an *incomplete or arrested histological differentiation* as compared with the foliage leaf[2]. On the other hand, the occurrence of specialised tissues, such as periderm, would appear to point to a *divergent* type of growth. It will now be realised that *these* observations regarding internal structure agree with Goebel's developmental characterisation of cataphylls as "arrested formations," *i.e.* organs which arise from the arrest and divergent growth of foliage-leaf primordia. More recent studies have been particularly concerned with this presumably dual explanation of bud-scale anatomy but, as will be shown, the emphasis has gradually shifted from the points of similarity between the scale and foliage leaf to the structural divergence between these organs. In the words of Droit (1908, p. 59) the similarity of bud scales to foliage leaves ceases with their external form for "leur structure microscopique s'écarte foncièrement de celle des feuilles ordinaires."

Thomas (1900 *a*), in a detailed study of the comparative and experimental anatomy of subterranean scale leaves in Monocotyledons and Dicotyledons has discussed a number of points which bear directly upon the problem of bud-scale morphology and the idea of "arrested formations." According to this investigator (*op. cit.* p. 422) subterranean scales are adaptive modifications of those portions of the foliage leaf nearest the axis. He recognised (*loc. cit.*) three general types of cataphylls, viz.

1. Those corresponding to the sheath of foliage leaves.

[1] Anderson (1897), among others, has reported stomata on bud scales, and Brick (1914, p. 223) only found the first of these negative features confirmed by his own extensive investigations.
[2] *E.g.* the frequent absence of palisade tissue. Cf. *e.g.* Coulter, Barnes and Cowles (1911, p. 642).

2. Those corresponding to the petiole or its sheathing base.

3. Those corresponding to the laminae of sessile leaves.

Thomas compared the anatomy of the scales with the corresponding leaf regions and his main results deserve brief summarising:

1. Stomates and hairs are usually reduced or even absent from cataphylls; if stomates are present, they tend to be more abundant on the upper surface of these organs (cf. Thomas, 1900 b, p. 89). The lower epidermis is usually heavily cuticularised.

2. Collenchyma disappears or at least is greatly reduced in the scales.

3. Palisade tissue is absent from the scales in the great majority of cases; he concluded (Thomas, 1900 a, p. 425) that while its presence or absence in cataphylls may be influenced by light, heredity plays the chief rôle in the development of this tissue. He found on the contrary that the parenchyma of the scales is more or less homogeneous and that air spaces are frequently absent.

4. The vascular structure of subterranean scales is reduced as compared with that of the foliage leaf, both as regards the number of elements of the xylem and phloem and the relative degree of lignification of the tracheary cells. Sclerenchyma is likewise reduced or absent from the cataphylls. With the exception of *Saponaria officinalis* L., a cambium and secondary tissue are absent in the vascular bundles of scales. (Cf. Droit, (1908), for a similar observation on the bud-scales of *Syringa vulgaris* L.)

5. There appears to be little regularity in the occurrence of secretory tissue, for according to the species, it may be more or less developed in the scale than in the leaf.

6. Reserve food materials are particularly characteristic of the subterranean scales of Monocotyledons and many Dicotyledons.

Thomas (1900 a, pp. 402–404) also investigated several species having both aerial and subterranean cataphylls. He found that in *Monotropa Hypopitys* L. the subterranean scale differs from the aerial scale in (1) the smaller extent of its parenchyma, (2) the larger size of its cells, and (3) the arrested development of its vascular tissue. In *Asparagus officinalis* L. the subterranean scale is distinguished by (1) the heavy cuticularisation of the lower epidermis and the absence of stomates there, and (2) by the decreased development of its vascular system.

Thomas devised two different sets of experiments to test the influence of the environment on the form and structure of foliar organs. In the first series of experiments (1900 a, pp. 419–420) young aerial shoots were placed beneath the ground for a period of time varying from one to two months; the leaves produced under the changed environment were scale-like in form in a number of cases, and were similar anatomically in many respects to cataphylls. Particularly in the case of *Saponaria officinalis* L., Thomas (1900 a, p. 430 and 1900 b, pp. 79–80) found that the leaves experimentally caused to develop below ground resembled the normal cataphylls in their thickened epidermal walls and in possessing reserve food materials; *etiolated leaves* of this plant, however, *closely resembled immature foliage leaves in their structure.* Conversely, Thomas (1900 a, pp. 420–422) found that when rhizomes of a number of species were placed in the light for about a month,

the upper cataphylls were transformed into organs practically identical in form and structure with the normal foliage leaves.

Thomas (1900 *a*, p. 429) pointed out that a number of the anatomical features of subterranean cataphylls are likewise peculiar to the early developmental stages of foliage leaves so that one might say that the scale only represents a leaf arrested in development. On the contrary, however, he concluded (*op. cit.*, pp. 430–432) that while *negative* characters of arrested development (such as the decrease in the number and size of the fibrovascular bundles, the reduction of the palisade and spongy parencyhma and the decreased prominence of collenchyma) are alike found in the subterranean scale and the young foliage leaf, *positive characters*, such as the increase in thickness of the epidermal walls and the production of reserve food material are absent both from the normal and the etiolated foliage leaves, and are alone found in the cataphylls; these later characteristics are regarded by Thomas as due to the peculiar environment of the cataphylls and to their special functions.

The work of Brick (1914) is of great importance, for he has utilised many of the anatomical facts brought forward by the Marburg School of Botanists in his detailed investigations of the comparative anatomy of bud scales and foliage leaves.

This investigator, with nearly the same developmental viewpoint as Goebel, classified (*op. cit.* p. 215) bud scales according to their origin from either (1) the primordium of a whole leaf, (2) the primordium of a leaf base, (3) the primordium of a leaf base with incompletely differentiated stipules, or (4) the primordium of stipules completely differentiated from the leaf base. Brick's third "group" includes the fulcreal scales of the earlier investigators, and as we have seen is not fundamentally distinguished by Goebel from the leaf-base type.

Brick confined his attention to "typical" representatives of the first three morphological groups of scales given above, and compared in great detail the anatomy of the successive scales of the bud with the region of the foliage leaf represented by them. In contrast to older investigators, who apparently only compared the structure of the *outer* bud scales with the foliage leaf, Brick compared the anatomy of the successive scales of the bud and, following Goebel's theory of "arrested formations," in all cases investigated that stage in the development of the foliage-leaf primordium at which *divergence* presumably occurs on the one hand to the mature foliage leaf, and on the other, to the mature bud scale. The comparative investigation was made in two ways (*op. cit.* p. 211):

1. In the first place, the part of the developing foliage leaf, at a stage in development at which a corresponding leaf primordium becomes directly modified to a bud scale, was compared with the mature bud scale.

2. In addition, that portion of the mature foliage leaf, whose modification is represented by the bud scale, was compared with the mature bud scale.

Brick concluded (*op. cit.* p. 298) from his studies that the *oldest* outermost leaf primordium of the winter bud is almost identical, morphologically and anatomically, with the youngest bud scale, so that these innermost scales may be properly considered as simply "arrested formations of foliage leaves." He based this conclusion not only upon the similar *quantitative development* of the mesophyll and the vascular

bundles, but also upon the more or less far-reaching *qualitative similarity* in the tissues of the innermost scales and the first leaf of the bud. On the other hand, he stated (*loc. cit.*) that the *outer* bud scales cannot be regarded merely as arrested formations, for changes appear in their development quite unlike those obtaining in foliage-leaf ontogeny; these outer scales, although "leaf-like," are "divergently developed organs." (Brick noted (*loc. cit.*) that the anatomical differences in the inner and outer scales are responsible for the divergent views expressed by Wiesner (*op. cit.*) and Droit (*op. cit.*).) *Qualitatively*, this is shown by the presence of corky, metacutinised and metadermised tissues. *Quantitatively*, according to him, the amount of mesophyll present in the outer scales tends to be greater than in the inner ones, while the converse is true, in most cases, of the vascular tissue. Brick (*op. cit.* pp. 214–215) maintained that the divergent qualitative development of the outer scales is due to the greater "plasticity of their cells at the beginning of the divergence, since they have been arrested at an earlier developmental stage than the inner scales." He also emphasised two hitherto neglected facts (*op. cit.* p. 299), *i.e.* that the *quantitative development* of bud scales and foliage leaves is more similar the nearer these organs stand to each other on the axis, and that the divergent development of the outer scales is accentuated by the further growth of the foliage leaves in the spring. It is further important to note that Brick (*op. cit.* p. 231, Table I) found in general that similar anatomical features may appear in the three morphological "groups" of scales investigated, thus lending support to the idea of Goebel and his school that the anatomical structure of an organ is independent of its morphology.

Neese (1916) in the first part of his paper discusses the differences between the leaf and flower buds in a number of woody species and homologises their bud scales with various regions of the foliage leaf. The second section of his paper is devoted to an exposition of the anatomical changes progressively exhibited in passing from the bud scales to the leaves and from the latter to the bracts. The main principle of his work is based on the fact that since the bracts and transitional forms are to be compared with the lamina of the foliage leaf "die Übergangsformen an der Sprossbasis und ebenso die Hochblattformen der Laubblattspreite homolog sein müssen" (*op. cit.* pp. 157–158). Even in the case of *Rosa* and *Rubus*, where the bracts are mostly leaf base in nature, the small apical laminae were alone used for comparison by Neese.

Neese argued that the structural characteristics of the lower bud scales and the foliage leaves might be expected to occur in a transitional condition in the intermediate forms between these two organs. He found that in the *lower portion* of the "transitional series" salient scale characteristics disappear, *i.e.* the leaves become thinner, the cell walls of the epidermal and "ground" tissues gradually decrease in thickness, small air-spaces appear, the large air cavities decrease as well as the crystal druses, and the cell size increases. Conversely, in the *upper portion* of the "transitional series" the anatomical features of the leaf tend to appear, *i.e.* the differentiation of palisade and spongy parenchyma (with an increase in the "mesophyll quotient"—*i.e.* the ratio of palisade to spongy parenchyma), an increase in

chlorophyll content and an increase in the number of stomates on both sides of the organs.

However, according to Neese, a number of structural modifications appear in the transitional forms which are found neither in the lower bud scales nor in the foliage leaves. He found particularly, that while in many plants the *upper surfaces* of the scales and leaves possess few or no stomates, the number of ventrally placed stomates is relatively large in the intermediate forms; in such cases, palisade tissue is usually absent, at least as long as the number of ventral stomates exceeds the steadily increasing number of dorsal stomates. Similarly, according to this worker, the degree of pubescence and the sinuousness of the radial walls of the lower epidermis tends to be more prominent in the transitional forms. Neese (*op. cit.* p. 181) emphasised that the above-mentioned anatomical features reach their *maximum* expression in the "transitional region," and hence do not show a progressive development towards the condition in the foliage leaf.

Neese similarly maintained that one might expect a corresponding anatomical reduction and simplification in passing from the foliage leaves to the bracts. As a matter of fact, he found that while the thickness and cell size of the successive bracts decrease, the "mesophyll quotient" does not remain the same, for the spongy parenchyma tends to increase. Furthermore, in many instances, the number of ventral stomates is greater and the sinuousness of the radial epidermal walls as well as the pubescence increases the higher the bract stands on the axis. Dorsal stomates also increase up to the leaf and then decrease. Neese (*op. cit.* p. 182) considered that these characters indicate the great similarity between bracts and the "basal transitional forms."

In attempting to interpret his observations, Neese (*op. cit.* p. 183) stated that external factors, such as light, can only be of secondary importance in causing the frequent reversal in orientation of the stomates, since he found that just as many ventral stomates appeared on the basal transitional forms in buds of *Syringa*, *Ligustrum* and *Lonicera* expanding in the dark as in the light; he assumed (*op. cit.* pp. 183–184) that the other characters peculiar to bracts and intermediate forms may be likewise performed in the bud. Furthermore, although the bracts and transitional forms show many of the characteristics of "shade leaves" (cf. Nordhausen, 1903, 1912), the bracts, at least in many cases, are better illuminated than the foliage leaves and the basal transitional forms. That the enumerated peculiarities of hypsophylls and transitional forms are not found in the "primary leaves," was checked by Neese for *Syringa vulgaris* L. and *Ligustrum vulgaris* L.

Neese (*op. cit.* pp. 185–186) after an anatomical study of the laminae of the calices of *Mespilus germanica* L. and *Rosa* maintained that here also the structural peculiarities, which quite resemble those of bracts, are determined not by the function nor the influence of outer factors on the organs, but that "inner factors alone come into consideration, which are obviously connected with the developmental seriation of the leaves at the growing point, *i.e.* with the leaf size; we should therefore assume that the leaf size also plays a rôle in the structural peculiarities. of the basal transitional forms and hypsophylls."

The fact that the basal transitional forms and the bracts either possess structural characteristics peculiar to them or reaching their *maximum expression* there as compared with the foliage leaves led Neese (*op. cit.* p. 182) to conclude: "Thus not only the outer scales, as Brick supposed, but also the transitional forms at the base of the shoot and the hypsophylls show a specific "divergent development" as compared with the foliage leaf: they are *not real arrested formations*, nor are they stationary stages of foliage-leaf development, but they have taken a developmental path which diverged from that of the foliage leaf at a definite stage and led to a distinct, divergent foliar structure."

The purpose of Fricke's (1926) investigations was to determine to what extent bracts differ structurally from foliage leaves, and whether these differences may be interpreted on the grounds that hypsophylls are "arrested formations." In contrast to Neese, he studied exclusively the bracts of herbaceous plants and devoted less attention to the transitional series. In general, he selected plants for investigation in which the bracteal and foliage-leaf regions are more or less sharply delimited. Fricke's method (*op. cit.* p. 250) differed from Neese's in that the bracts, *regardless of their morphology*, were directly compared with the foliage leaves; a procedure justified on the grounds that since the anatomy of an organ is independent of its morphology[1], "Absolute Gesetzmässigkeiten müssen daher im Gegenteil eher noch leichter an ungleichmässigem als an homologem Material sich feststellen lassen."

On the basis of a comparative study of the bract and foliage-leaf anatomy of about 37 species of Angiosperms, Fricke (*op. cit.* pp. 280–281) maintained that the anatomy of a foliar organ is independent of its morphology and of any "arrest" in its development. The mesophyll of a bract for example may (1) remain quite simple, or (2) differentiate like that of the foliage leaf, or (3) develop in an entirely new direction. Similarly, according to Fricke (*op. cit.* p. 281), many other structural peculiarities of the bract, such as the distribution of the stomates, the type of epidermis, the pubescence, the structure and course of the vascular bundles, etc., are unrelated to an arrest in its development. Fricke (*op. cit.* pp. 281–282) also found that none of the regularity in the expression of anatomical structures, emphasised by Neese, occurred in his own material, and further that hypsophylls do not exhibit the structural features of "shade leaves" (cf. Nordhausen, 1912). In short, Fricke (*op. cit.* p. 282) held that neither a comparison of the *common structure* of bracts nor an examination of their *individual characteristics*, *i.e.* as contrasted with the foliage leaf, provide an explanation for the appearance of the "neu Merkmale" of hypsophylls.

Fricke then attempted to analyse the "causal" factors regulating the divergent structure of bracts (*op. cit.* pp. 283–286). The conditions of nutrition, he believed, could only modify the structure of hypsophylls according to the particular "plan" of the individual plant. Furthermore, whilst a more or less definite correlation exists between the structure of a bract and its relative size (cf. also Neese, *op. cit.*

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[1] This represents a general canon of the Goebelian school of morphologists. For a criticism of this viewpoint, cf. Arber (1925, pp. 4–6).

p. 185) or position on the plant, we gain little from this fact alone because "correlations are only the expression of causes." In this connection Fricke pointed out that characters which appear "new" in a bract (in contrast to the foliage leaf) are not necessarily "specific" but indeed may also appear in the cataphylls or stipules of the same plant—*i.e.* in organs which obviously are related to entirely different inner factors. Lastly, although the bracts in many instances are of "use" to the plant as "display" or protective organs, one should not account for their presence merely on teleological grounds—for in addition to characters of ecological significance there are often those which are quite non-essential.

On the contrary, according to Fricke (*op. cit.* pp. 285–286) "the plant in its ontogenetic development always forms those organs and characteristics which *must* develop under the influence of the actually prevailing inner conditions," *i.e.* "without reference to their usefulness and simply according to the potentiality of the specific structure of the individual plant." In spite of the difference in his material and observations, Fricke (*loc. cit.*) concluded like Neese that: "The bracts are divergent foliar structures, and not arrested stages of foliage-leaf development, and inner factors should be made responsible for the nature of their characteristics." (Küster (1925, p. 288) regards the incomplete differentiation of the mesophyll of many cataphylls and hypsophylls as an example of a "hypoplasy" determined by the influence of "inner factors.")

Anatomical studies, particularly those of Thomas, Brick, Neese and Fricke, have given us a viewpoint regarding the real nature of bud scales and bracts which appears fundamentally unlike Goebel's theory. The root of this difference undoubtedly lies in the meaning of the expression "arrested formations[1]." As we have already seen, Goebel regards an "arrested formation" in the sense that at an earlier or later stage, a foliage-leaf primordium experiences an arrest in its development which is usually followed by a divergent growth of one or more of its parts. That a corresponding change in internal structure may occur is indicated by Goebel's observations (1908, pp. 32–34, and Fig. 15) on the scale leaves of *Veronica cupressoides* which he considered, like bud scales, as "arrested formations." He found that these scale leaves possess assimilating parenchyma next to the *dorsal surface*[2], whilst in the "juvenile leaves" this tissue is ventrally directed; stomates are also exclusively found on the dorsal epidermis of the scale leaf, whereas they occur on both surfaces of the "juvenile leaves." Goebel attributed the stronger development of the dorsal side of the scale leaves indirectly to their vertical position which results in changed conditions of transpiration and illumination (as compared with the horizontally placed "juvenile leaves") and the accumulation of organic materials on their lower surfaces. Goebel is thus inclined to interpret the divergent anatomy of such organs as a result of external conditions, a view which Thomas (1900 *a*) also held regarding subterranean cataphylls (cf. Küster, 1925, pp. 255–288,

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[1] My analysis of this difficult question has been greatly aided by extensive private correspondence with Dr Georg Fricke, who has taken great pains to clarify both his own viewpoint and that of the late Dr Neese.

[2] Adlerz (1881) had already noted that palisade-like cells appear on the dorsal side of the bud scales of *Elaeagnus argenteus* and *Hakea corymbosa*.

for a detailed discussion of the quantitative and qualitative "hypoplasy" of tissue in relation to external factors. The extensive literature on the subject is cited).

Brick's investigations were more far-reaching in that he attempted to correlate the *quantitative* and *qualitative anatomical differences* between the inner and outer scales of the bud with the time at which the "arrested development" presumably occurred—under his theory, the "arrest" of the outermost primordia of the bud takes place at a young and "plastic" stage in ontogeny, and hence "divergently developed organs" are formed.

Neese and Fricke, however, found no evidence to support a "dual" interpretation of bud scales and bracts—they have really substituted the expression "individuelle Blattgebilde" for Goebel's term "Hemmungsbildungen." The word "individuell" is used by these investigators in a *colloquial* (not a philosophic!) *sense* to emphasise the *divergent structure* of cataphylls and hypsophylls[1]. Fricke (*op. cit.* p. 280) in particular interprets "Hemmungsbildungen" as meaning that "only the *normal* course of leaf development becomes arrested." He looks upon the ontogeny of a foliar organ, such as a scale or a bract, as a "*smooth*" process which cannot be sharply divided into a period of arrest followed by one of divergent growth. That is, the bracts usually do not *cease* growth at an early period, but rather experience development along a different path than that taken by the foliage leaf. Exceptions, according to Fricke, only occur (1) when bract development is *completely* arrested, or (2) when the hypsophylls are only distinguished from the foliage leaves by their position, or (3) when the structural characteristics of the foliage leaf appear in a less definite expression in the bracts (cf. Fricke's, *op. cit.* p. 258, remarks on *Lythrum salicaria* L.).

Fricke's theory is supported in still another direction. He found (cf. p. 276) that the same plant can produce several types of bracts, each with very different internal characteristics, as in *Dalechampia* and *Schizocapsa*. The prophyll of *Lallemantia*, *e.g.* with its bristle-tipped lobes, cannot be an "arrested formation" for "the history of development of the foliage leaf showed that only rarely are single short leaf teeth visible in the younger stages[2]." Fricke (*op. cit.* pp. 266–267) made the further interesting observation that in *Fittonia gigantea* Lind. the bract morphologically represents about equal parts of the "lower" and "upper leaf" of a foliage-leaf primordium—both regions of the bract, however, are structurally different.

It is also of interest to note the interpretation which has been placed on the so-called "transitional forms." These organs were regarded by Neese as "individuelle Blattgebilde" since in *his* material they represented the place of maximum expression of ventral stomates, sinuous epidermal walls etc. Fricke does not fundamentally distinguish bracts from the intermediate forms—the latter he regards (*op. cit.* p. 277)

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[1] Dr Fricke wrote me: "Im deutschen *Sprachgebrauch* dagegen wird 'individuell' angewandt als Fremdwort in der Bedeutung von besonders, abweichend, bestimmte Eigenschaften besitzend, gekennzeichnet."

[2] Glück (1919, pp. 280–286) has described a number of cases of highly segmented bracts, and Goebel (1923, pp. 1401–1402) also noted that hypsophylls, in some cases, are more segmented than the foliage leaves, but gave no evidence to show that such structures are "arrested formations."

as "mixed formations" between foliage leaves and bracts. In particular, he believes it quite possible that the outer involucral leaves of the Compositae may be comparable to the "basal transitional forms" of the vegetative shoot in that they have characteristics *sui generis*.

The discrepancies in Neese's and Fricke's observations *may* be referable to a fundamental difference in the organisation of the leafy organs of herbaceous and woody plants—more evidence is needed, however, before this suggestion becomes at all probable. It should be realised, however, that both of these investigators dealt largely with *mature organs*. A more critical judgment of Goebel's theory of "arrested formations" can only be attempted when more precise information is available as to the *time of origin* of anatomical structures in the foliage leaf as compared with the bract or bud scale. Nevertheless, the work of Neese and Fricke has definitely shown that many of the external and internal characteristics of cataphylls and hypsophylls cannot be explained in terms of a simple theory of the arrest and subsequent divergent growth of foliage-leaf primordium.

6. THE PROBLEM FROM A "CAUSAL" STANDPOINT.

(i) *The Nature of Leaf Primordia.*

It should now be evident that the problem of bud-scale morphology, from its early phases up to the present, has been dominated by the Doctrine of Metamorphosis in some form or other. Many of the early botanical writers used the term "metamorphosis" more or less vaguely, to express the idea that the form of "something" has been changed. We have already seen that under the idealistic morphology, this "something" was the abstract "Urblatt" which has no concrete existence, but simply appears in various degrees of "transformation" as the manifold "leaves" of the shoot. Other writers have preferred to think of "metamorphosis" as a phylogenetic process, whereby the diverse leaves of modern plants have arisen historically from a common "type" such, for example, as Prantl's (1884, p. 55) "Laubsporophyll"—this viewpoint is usually supported by teratological observations and by the *assumption* that the ontogeny of the plant *as a whole* frequently recapitulates its phylogeny. Any evolutionary concept of "metamorphosis" however, under our present dearth of knowledge regarding the nature of the primitive Angiosperm leaf, is capable of no *inductive* proof and appears largely speculative in nature.

Obviously, "metamorphosis" can never have the concrete meaning in botany that it has in zoological science. In the metamorphosis of insects, for example, the same individual during its ontogeny passes through a number of more or less separate and distinct form changes, with corresponding differences in functional activity. On the other hand, the growth of the higher plants is essentially a continuous process during which large numbers of morphologically homologous organs, *i.e.* the "leaves," are formed serially by the activity of the meristematic growing-points. Under such circumstances, the seriation in *mature* form of the leaves on the shoot is surely not referable to a "metamorphosis." We have to deal rather with "epigenesis" or a process of organ differentiation at the growing point. In

short, the "leaf" shows no functional "larval stages" analogous to those of animals, but its ontogeny is virtually an uninterrupted series of developmental stages.

Although the value of the ontogenetic method in the study of leaf form has become increasingly evident in recent years, the problem of foliar differentiation itself seems as perplexing as ever. In the writer's opinion, progress in this field will only be realised when we have more clearly visualised the problem from the standpoint of the form changes taking place at the growing point. On the one hand, considerable attention has been devoted in the past to the history of development of foliar organs. On the other hand, less but equally valuable study has been made of the relation of various factors, such as light, nutrition, etc. to leaf ontogeny. These two methods of investigation, however, ultimately converge upon a common unit—the "leaf primordium" which, in a general way, may be compared to Eichler's unsegmented "primordial leaf." Whilst any definition of "leaf primordia" is necessarily artificial at present, these exogenous structures *per se* are primarily characterised by two features, viz. (1) they are meristematic, *i.e.* relatively "undifferentiated" internally and hence particularly sensitive to the external and internal conditions prevalent during their growth, and (2) they are composed of cells with a specific genetical constitution. In spite of these facts, several investigators have interpreted foliar anlagen solely in terms of the adult organs "normally" arising from them (cf. Goebel, 1900, p. 8). Thus the "Congenital Theory," which has been previously outlined, lays particular stress on the *specific individuality* of the primordia—in Worsdell's opinion (1916, p. 122) "a stamen is a stamen and nothing else from its birth onwards." Considerable doubt arises as to the correctness of this theory in the light of experiment[1] and teratological phenomena. Under the "normal" conditions of growth, the primordia at the base of the axillary shoot in woody plants usually develop in a definite fashion to bud scales—any disturbance of the delicate metabolic relationships, however, such as that produced by injury, defoliation, or exceptionally vigorous growth, indicate that these organs are not necessarily strictly limited in their developmental possibilities, even after considerable differentiation has taken place. Schneider (1913), for example, found that the usual number of needles on the spur-shoots of *Pinus Cembra, Strobus, aristata* and *sylvestris* may be augmented by the "metamorphosis" of the scales of the cataphyllary sheath or of the terminal bud. He observed that the normal anatomical differences between scale and needle (*i.e.* absence in the former of stomates, layered assimilating tissue, transfusion tissue and an endodermis) are completely equalised after the "transformation" has been completed. Losch (1916) observed that the innermost scales of opening terminal buds, on trunk sprouts of *Aesculus hippocastanum* L., although morphologically equivalent to leaf sheaths, developed apical leaflets up to 9·4 cm. in length and persisted apparently as photosynthetic organs until autumn[2]. He found that this phyllody is accompanied, in the sheath portion of these structures, by a curious elevation of the stomates and the formation of

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[1] Cf. Goebel (1880, 1884), Pfeffer (1903, pp. 141–145) and Klebs (1904, p. 292).

[2] This phenomenon had been previously recorded by Pluskal (1854). Perriraz (1910) noted a similar condition on vigorous shoots of *Fraxinus ornus* as well.

callus-like processes in the schizogenous air cavities. Losch interpreted these anatomical changes as a direct adaptation on the part of the scale to conditions of increased transpiration. (Gluck, 1919, pp. 358–359, uses teratological data to support his belief that sepals are "arrested formations" of foliage leaves.)

In contrast to the "Congenital Theory," Goebel maintains that the plant only forms *one* type of "leaf" initial, *i.e.* foliage-leaf primordia. Under his theory, the foliage leaf represents the end-product of a causally related series of developmental stages which are "inherent" in the primordium—all other foliar organs, such as cataphylls or bracts, result from an earlier or later arrest and divergence in the developmental history of the foliage leaf. Is it quite correct, however, to emphasise the *dominance* of foliage-leaf characteristics in every primordium? Furthermore, why is it necessary to interpret cataphylls as a divergence from an "inevitable" chain of developmental stages leading to the foliage leaf? It is clear from the work of Neese (1916) and Fricke (1926) that the internal differentiation of cataphylls and hypsophylls certainly does not parallel that of the foliage leaf in many cases— a similar lack of agreement in external form development is not of infrequent occurrence, as will be pointed out later. By a curious paradox, Goebel in his efforts to be concrete, has abstracted the developmental characteristics of the foliage leaf and transferred them to every leaf primordium. It seems perfectly clear, however, that the "typical" foliage leaf, whose appearance is just as definitely "regulated" as that of the bud scale, only represents *one* of the many developmental possibilities of the leaf primordium.

Although the "Differentiation Theory" as advanced by Frank (1893) is an attempt to avoid the difficulties apparent in Goebel's assumptions, it fails to make clear the meaning of "indifferent" leaf primordia. Pfeffer (1903, pp. 142–143) defined all primordia of generalised character and powers as "indifferent, neutral or indeterminate." In his opinion, such primordia only subsequently become *determinate* "by the partial suppression or modification of their generalised embryonic properties," and they may then be distinguished as the anlagen of leaf, root or stem. (This viewpoint is directly opposite to Goebel's (1900, p. 8) belief in the fundamental individuality of leaf and stem primordia.) Pfeffer writes: "Naturally all specific leaf primordia or stem meristems are equipotential among themselves, although they may undergo dissimilar differentiation under varied conditions." Indeed, the type of development of a leaf primordium seems to depend on at least three factors, viz. (1) the genetical constitution of its cells, (2) the kind of growing point with which it is associated, and (3) the external and internal conditions obtaining during its ontogeny. Since these factors are *always* operative, the leaf-primordia, at least in the "post-embryonic" period, are *not* indifferent, but as Berthold (1904, p. 2) held, are possessed of definite specific characteristics.

The problem of foliar differentiation has taken on a new and broader aspect as the result of the experimental work of Klebs. This investigator published in 1903 the results of his studies on the artificial production of "transformations" in floral organs. In contrast to Goebel, Klebs (1903 *b*, pp. 4–6) regarded the causes of "metamorphosis" as more important than the *functional change* involved. He

rejected Goebel's idea of a "real metamorphosis" (except in a figurative sense) and used "metamorphosis" simply as a "convenient expression without the slightest pretension of wishing to 'explain' anything thereby." (Glück (1919, p. 343) maintained that Klebs only "replaced" Goebel's theory with a difficult conception!) One must assume, according to Klebs, that in the young growing primordium of an organ, a number of developmental potentialities exist; under the "normal" external conditions of the flower, one potentiality, *e.g.* that of the stamen, may dominate in a given primordium, whilst the others remain inactive. Under certain modified inner and outer conditions, the dominant potentiality corresponding to the place of origin of an organ may become ineffective and completely replaced by another "Potenz," or two or more potentialities may be simultaneously expressed[1]. In order to determine which dominant potentiality (Hauptpotenz) has been assumed, we must know the number and arrangement of the organs, supported by the history of their development. Further experimental studies, which will be mentioned later, strengthened Kleb's belief in the extremely "plastic" nature of young leaf primordia.

The evidence at hand thus appears to suggest that the leaf primordia at the growing point of the shoot are genetically *multipotent* and hence capable of development along a number of lines which are secondarily determined by external and internal factors, and by the relation of the growing initial to the shoot as a whole. From this viewpoint, we are now in a position to re-examine the "formal" and phylogenetic aspects of the problem of bud-scale morphology.

(ii) *A Re-examination of the Formal Aspects of the Problem.*

As we have already seen (cf. pp. 127–130) the formal viewpoint of the problem is based on the supposed existence of one or more of the "elements" of the foliage leaf, in a "transformed" or modified condition, in the bud scale. Since the earlier work in this field was largely based on a comparison of adult structures, many errors in interpretation naturally occurred, some of which have been corrected by developmental studies. It is now perfectly obvious, for example, that any classification of buds based on scale morphology is artificial and in many cases quite out of the question (cf. Löfling (1751), De Candolle (1841) and Henry (1846)). Brick (1914, p. 217) has emphasised that the same species may have bud scales representing several "morphological groups." He found (*op. cit.* p. 270), for example, that three "morphological groups" are in turn represented by the successive scales on the bud axis of *Crataegus Crus Galli*.

However, the question may now be fairly asked: Is a developmental classification of bud scales into more or less distinct "morphological groups" possible or even desirable at the present stage of our knowledge? Goebel's investigations seemed to answer this question in the affirmative, at least for a limited number of species[2],

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[1] A similar idea has been recently advanced by Haecker (1927, pp. 52–54) who regards foliar anlagen as "isopotent" and points out the relation of this conception to Goethe's interpretation of "metamorphosis."

[2] It seems clear from his recent publications that Goebel has extended his theory to few, if any, species not already discussed previously in his paper of 1880.

but in the present writer's opinion, the specific morphology of bud scales, in a large number of instances, must be viewed in a somewhat different light.

As early as 1846, Wigand (pp. 6–8) expressed the opinion that the attempt to homologise the various portions of "transformed" organs with regions of the foliage leaf was based upon the assumption of a *material metamorphosis*. Goebel has tried to avoid such an abstraction by advancing a theory of the bud scale based upon three premises, viz. (1) that cataphylls arise by a "real metamorphosis" of foliage-leaf primordia; (2) that Eichler's theory (with some modification) of the segmentation of the "primordial leaf" into "leaf base" and "upper leaf" represents a widespread developmental characteristic of foliage leaves; and (3) that therefore the specific homologies of cataphylls are referable to an arrest and modification of the developmental history of the foliage leaf. Reasons have already been given for the improbability of Goebel's first premise. The attempt will now be made to show that his last two premises also fail to account for the morphology of the scale in many instances.

Goebel (1884, p. 246) assumed that the absence of a petiole is a characteristic feature of Dicotyledonous bud scales. In a number of plants such as *Acer* (cf. Pax, 1885, p. 299), *Aesculus* (cf. Pax, 1897, p. 273), *Mahonia* (cf. Fedde, 1901, p. 39), etc., the bud scales appear quite clearly to represent a modified development of the basal portion of the primordium with a concomitant "arrest" of the laminar region. That a "petiole" is not invariably absent from cataphylls of this type, however, is shown by Berthold's (1904, p. 31) observations. He found that two types of "transitional leaves" occur in the bud of *Acer pseudoplatanus* L. In the "lower" intermediate forms appearing in opening buds the lamina is directly seated upon the leaf base and the petiole is absent, whilst in the outermost scales of the terminal bud, all three "elements" of the leaf appear. (Losch (1916, pp. 682–683, Figs. 7 and 8) made a similar observation for *Aesculus hippocastanum* L.) Berthold noted that the laminae of these latter scales usually fall during the summer. The present writer has found a similar situation in other plants, such as *Fraxinus*. From these examples alone, we should be warned of the danger of constructing hard and fast "morphological" definitions of bud scales.

It follows from Goebel's developmental viewpoint that the lamina or "Oberblatt" of the foliage-leaf primordium should always be present to some degree in the bud scale. For example, Goebel (1884, p. 244) strongly emphasised in contrast to the statements of Henry (1846), Döll (1848) and others, that the corresponding laminae of the stipular bud scales of *Quercus*, *Fagus*, etc. are always present as unstalked pointlets. Kŏstál (1903) has also shown that in *Alnus* and *Betula*, the laminae associated with the outermost stipular bud scales remain microscopically small. Schmidt (1889, p. 15), however, was unable to find any laminar rudiments in connection with the lower stipular scales of *Carpinus betulus* L., *Fagus silvatica* L., *Quercus pedunculata* Ehrh., and *Q. sessiliflora* Salisb., and concluded that the blade here is "completely aborted." The need for more careful developmental work in such cases is evident. Furthermore, whilst the stipular type of cataphyll may often resemble the foliage leaf in development, this certainly

is not inevitably true. Langdon (1927) in a recent study of the seedling buds of *Quercus alba* L. and *Q. rubra* L., found that whilst the foliage leaves are tri-lacunar (cf. Sinnott, 1914), the stipular scales are "bilacunar." It is clear from her drawings that this difference is ontogenetically fundamental and suggestive of the fact that the history of development of the cataphylls here is initially unlike that of the foliage leaf.

Goebel's theory, however, experiences one of its greatest difficulties when applied as an interpretation of *unsegmented* cataphylls or hypsophylls. The problem in these cases definitely settles upon the correctness of Eichler's view regarding the uniformity in the primary segmentation of Angiosperm leaves. Schmidt (1889) in particular, has taken up this matter in regard to the comparative ontogeny of hypsophylls. He found that in the majority of the plants investigated possessing well-defined leaf sheaths or stipules, the bract corresponds to the sheath whilst the lamina is rudimentary or absent. In Phaseolus, in a number of the *Caryophyllaceae*, etc., Schmidt found no indication of the differentiation of an "upper leaf" in the bracts. Goebel (1923, pp. 1402–1404) interprets such cases as due to an "arrest" of the foliage-leaf primordium prior to its separation into "leaf base" and "upper leaf"—bracts (and cataphylls) arising in this way are thus "sheath-like structures with no indication of a blade." Schmidt (*op. cit.* p. 11) on the contrary held that such hypsophylls are equivalent to *unchanged* leaf sheaths with *completely* arrested laminae. In the present writer's view, either interpretation leads to abstractions—the important fact seems to be that the bracts and cataphylls in such cases do not parallel the foliage leaf in development. Schmidt also found that in plants with exstipulate or sheathless leaves, the leaf primordia do *not* segment into a "leaf base" and "upper leaf"—the bracts in such cases, although sheath-like in form, seemed to him to represent modified and broadened "leaf blades." (Cf. Goebel's (1923, p. 1400, Fig. 1336) directly opposite remarks on the bract of *Rhinanthus*.) Goebel (1880) originally interpreted many bud scales in this way, but in the recent edition of his *Organographie* (Goebel, 1923, p. 1394) he refers such cataphylls to a "trans-formation" of the whole leaf primordium. Here as before, the attempt to distin-guish between a part of the "leaf" and the "whole" inevitably leads to abstractions. On the other hand, it is clear that Goebel's theory of "arrested formations" be-comes fantastic in cases where neither the foliage leaf nor the scale segments into "leaf base" and "upper leaf." Goebel (1880) himself was unable to account satisfactorily for the specific morphology of the cataphylls of *Picea*, *Paris quadri-folia* L., and *Oxalis* on his own theory.

Furthermore, Goebel's theory seems inadequate to explain the striking differences in the form and position of the so-called prophylls of axillary winter buds. Whilst the "prophylls" in many cases seem indistinguishable from the other bud scales except for their position on the axis (cf. Goebel, 1923, pp. 1391–1393), they fre-quently are unsegmented or exhibit some anomalous conformation. Goebel (1923, p. 1395) distinguishes the prophylls of *Quercus* from the upper paired stipular scales of the bud, as "simple structures." It seems unlikely that prophylls of this type can be interpreted as "arrested formations," for their development is probably

quite divergent from that of the foliage leaf. As a matter of fact, Goebel (1880, p. 808), in the course of his experimental studies on *Prunus Padus* L., only found the prophylls in rare cases developing to curious foliaceous members which bore little resemblance to a foliage leaf. To explain this he advanced the purely theoretical suggestion that the prophylls arise normally by a "metamorphosis" of *unsegmented* foliage-leaf primordia. The theory of "arrested formations" experiences a similar "strain" in relation to Rüter's (1918, pp. 257–259) work. By employing several experimental treatments she was only able to produce a greening and an enlargement of the scaly prophylls in a number of Monocotyledons. She concluded that the "transformation" of the primordia to prophylls here has occurred so early (!) in ontogeny that the induction of further development is impossible. There is evidence to show that in a number of cases, the prophylls are morphologically "double" in nature. For example, the single cap-like bud scale of *Salix*, which has long been regarded as a "double" structure, has been shown by Kõstál (1903) and Resvoll (1909, pp. 332 *et. seq.*) to arise by the ontogenetic fusion of two separate opposite primordia[1]. Whether the supposedly "double" nature of the prophylls of *Populus*[2], *Hedera helix* L.[3], and *Carya*[4] can be ontogenetically proved, remains a question at present. The morphological nature of prophylls can be discussed no further here—for more complete information regarding this controversial question reference should be made to the work of Weiss (1889, 1891, and 1899), Dutailly (1879), Bremekamp (1915), Rüter (1918), Collins (1924) and Arber (1925). However, the fact that prophylls are only associated with axillary buds (this fact was emphasised in 1847 by Henry) supports the argument previously advanced by the writer, *i.e.* that one of the factors regulating the development of a leaf primordium is its relation to the shoot as a whole, and the type of growing point upon which it arises. A developmental investigation of prophylls from this standpoint should be productive of results rather than of phyllotaxic speculations.

(iii) *Recapitulation and the Phylogeny of the Bud Scale.*

Lastly, the fact remains that the cataphylls and hypsophylls of many species exhibit a type of segmentation which so far has received no satisfactory developmental explanation. As early as 1844, Savi pointed out that the basal leaves of the shoots in *Aesculus hippocastanum* L. may have wing-like proliferations which he regarded as stipules. Later investigators, such as Rossman (1857) and Lubbock (1890, 1894, 1897, 1908) have recorded the presence of stipular wings or vestigial stipules in the bud scales of numerous exstipulate species without attempting more than a teleological explanation for the situation. A number of other writers, however, for example Tyler (1897), Velenovský (1907) and Domin (1911 *a* and 1911 *b*), on purely theoretical grounds, have attempted to "recover" the primitive form of Angiosperm leaves in the "cataphyllary series" of the plant. The general viewpoint of these workers is that bud scales have "retained" the original condition of the

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[1] Goebel (1888) also found the prophylls of *Scirpodendron* fusing during ontogeny.
[2] Cf. Döll (1848, p. 7), Pax (1894) and Weiss (1904).
[3] Cf. Weiss (1924).   [4] Cf. Döll (1848, p. 22) and Engler (1894).

stipules or sheath whilst these latter structures have been "lost" or modified in present-day foliage leaves. (Similar recapitulatory ideas of bud scales have been advanced by Fankhauser (1882, pp. 7–8), Cook (1916) and Dorsey and Weiss (1920, pp. 401–402).) A detailed consideration of the inconsistencies and specula-tions involved in the first three papers cited would carry us too far—besides, Schrödinger (1919) has already given a most able and just criticism of this work. Attention should be drawn to the fact, however, that according to this interpretation of the bud scale, the apical lamina when present is regarded as a *nascent* rather than as an arrested structure—this point will be taken up again later.

Goebel's theory of "arrested formations" found its warmest supporter in Glück (1919) who has applied it in his extensive reconnaissance as an aid in the interpreta-tion of the foliar organs in the Angiosperms. Glück is particularly concerned in his exhaustive monograph with the homology between stipular structures through-out the Monocotyledons and Dicotyledons, and with demonstrating the relative antiquity of the sheath as compared to "free" lateral stipules. His morphological interpretation is based upon an interesting combination of ontogenetic and "re-capitulatory" evidence. As the result of his study of the leaf forms in seedlings of *Potamogeton* and *Nymphaea*, he was led to conclude (*op. cit.* pp. 277–278) that lateral stipules are phylogenetically more ancient than a sheath, the latter having arisen by a "fusion" of the stipules with the base of the petiole[1]. In adopting this as a working hypothesis, Glück made the important observation that stipules or stipular appendages in many plants are confined to certain organs, such as hypso-phylls, sepals, petals or stamens, whilst the stipules are "represented" in the foliage leaves and cataphylls as simple sheaths. Since this fact could not be explained in terms of Goebel's theory alone, Glück was forced to an additional assumption. He maintained (*op. cit.* pp. 164–177) that the foliage leaf, which he held as the precursor of all "leaves," recapitulates its phylogeny during its various developmental stages, some of which find permanent expression in cataphylls, bracts, etc. as well as in the intermediate forms between these organs. Glück then consistently took the position that hypsophylls and floral organs very generally represent phylogene-tically *older* stages in leaf evolution than the cataphylls and the sheaths of the foliage leaves, *i.e.* phylogeny may be repeated in the bracteal region, for example, in an *inverse* direction as compared with the cataphyllary series. Glück interpreted the unsegmented cataphylls in many plants as "fused" stipules devoid of laminae—he could only account for the tendency towards "fusion" in the cataphyllary region on the basis of the "need" for broad protective structures about the growing point.

It must be admitted that Glück's conclusions cannot be lightly dismissed, for they are based upon a tremendous amount of new and valuable morphological data. In a recent summary of his earlier work, Glück (1925) confesses that ontogeny gives no evidence of the stipular origin of the sheath in Monocotyledons. He attempts (*op. cit.* p. 164) to justify his theory on the grounds that it allows one to bring

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[1] A precisely opposite conclusion was reached by Domin (1911 *b*) who based his idea of the primitiveness of the sheath largely on the ancient doctrine of Anaphytosis. Cf. Schrödinger (1919) for further details.

certain foliar organs into genetical relationship whose "morphological value" would otherwise remain quite obscure. The present writer is in no position to question the accuracy of Glück's observations. The point is rather: Has the application of the so-called law of recapitulation proved a trustworthy guide in phylogenetic questions in botany? A few investigations in this direction may be cited to show the controversial nature of the whole subject.

Massart (1894) interpreted the juvenile forms of many seedlings as simple adaptations to special functions. He explained the infrequency of recapitulation in plants as compared with animals on the grounds that the former are stationary and must become quickly adapted to a particular environment, whereas the animal in its early embryology usually occupies the environment of its ancestors. A similar idea was held by Bailey and Sinnott (1914, p. 47) who state: "However, the meristematic tissues of the developing plant are in all probability more subject to modifying environmental influences than are the embryos of the higher animals."

Goebel (1900, p. 143) recognised two types of juvenile development, namely, the homoblastic and the heteroblastic. Homoblastic development is illustrated by the seedlings of *Casuarina* in which the leaf configuration above the cotyledons is essentially the same as in the adult shoot. Heteroblastic development is represented in those Australian Acacias which possess phyllodes; here the primary leaves are frequently like the normal foliage in related species without phyllodes. Goebel says (*op. cit.* p. 144): "The retention by the seedling in this case of the original, phylogenetically older, form of the vegetative organs is connected with its living under other conditions than does the adult form." In other cases, Goebel (*op. cit.* pp. 170–171) maintains that the juvenile foliage has been changed by adaptation or simply represents "an *arrest* which is probably a consequence of relationships of correlation."

Dufour (1910) found that the form of the adult leaves of a given species may be represented by the juvenile foliage of the seedling in a related form, from which he concluded that recapitulation indicates the path of foliar evolution assumed by the various species of a genus. Although Tyler (1897, p. 26) considered that phylogenetic changes are disguised in the seedling, Dufour (*op. cit.* p. 382) maintains that the primary leaves "se forment généralement aux dépens des réserves de la graine, c'est-à-dire dans des conditions constantes."

Nicoloff (1910), although admitting that the biogenetic law is not universally applicable, found that the similarity of the primary leaves of a species with the mature foliage of a related plant is of great value in showing systematic affinities. Thus he regarded the pinnate leaves of *Acer negundo* L. and *Fraxinus excelsior* L. as phyletically "young" compared with the foliage of other species of their families, since their seedling leaves are simple[1].

An admirable discussion of the broad aspects of the questions of recapitulation

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[1] Lewis (1907, p. 441), however, interprets the formation of relatively simple leaves in plants bearing lobed or compound leaves "as an arrest of development in the primordial leaf, followed by a stage of expansion, or by expansion before the embryological stage has been completed. Rapidity of growth may account for the constant location of the simpler leaves near the cotyledons, bud scales and sepals."

and reversion is given by Shull (1905) in his memoir on "Stages in the development of *Sium cicutaefolium*." This investigator has made a careful survey of the frequency of occurrence of the extraordinarily polymorphic leaf types in the three supposedly "primitive" regions of this plant, namely: (1) in the seedling, (2) in rejuvenated buds, and (3) in the inflorescence axis. He found that whereas the "modal form" of the first nepionic leaf is trilobate, an *undivided* leaf usually appears at the second and third nodes, before the pinnate condition characteristic of the upper leaves is developed. (Such an unexpected "break" in the leaf series can scarcely be said to furnish support to the phylogenetic importance attached by Jackson (1899) and Cushman (1902, 1903, 1904) to "localised stages" in leaf development.) The "senescent" series in the inflorescence differs markedly from the juvenile condition in that a progressive reduction in the blade and petiole obtains. Shull points out (*op. cit.* p. 12) that this situation is not in accord with the idea that the senescent stages repeat phylogeny in an inverse order as compared with the nepionic leaves. Shull observed (*op. cit.* p. 18) that when young flower buds were rejuvenated by submergence in water, "the proliferations presented extreme juvenile conditions, showing several types of leaf which are simpler than any which were found in the seedlings, and usually reaching the condition of the first nepionic leaf at the third or fourth leaf of the proliferation." He suggested that both senescence and the stage of development reached by a leaf in "rejuvenescence" may be ascribed to the "relative value" of the fluidity of the protoplasm and the available "food-equivalent" rather than to phenomena of atavism. Since the theories of Tyler (1897), Domin (1911), Cook (1916) and Glück (1919) are really grounded on the assumption that the relatively unsegmented character of bud scales is phylogenetically significant, Shull's general conclusions (*op. cit.* p. 27) are of particular interest: "The sole basis for the assumption that localised stages[1] present atavistic characters is the fact of their greater simplicity. No satisfactory inferences can be drawn from ontogenetic leaf-characters regarding the phylogenetic history of the species. The various stages are the result of present protoplasmic structure instead of the past history of the protoplasm, and a change of structure, which results in new adult characters, may also produce changed juvenile and senescent characters. They are in need of physiological instead of phylogenetic interpretation." In the present writer's opinion, Shull emphasises a much neglected point in the above statement, *i.e.* there appears to be no sound *a priori* reason why certain foliar organs, such as bracts or scales, should pass through the ages relatively unchanged. On the contrary, such organs have undoubtedly had a long and involved evolutionary history of their own, which, however, at present is completely obscure to us.

The conclusion appears justifiable that the principles of recapitulation and reversion appear inapplicable at present for the determination of the palingenetic or coenogenetic nature of bud scales and bracts (cf. Diels, 1906, pp. 108–114). As Bailey and Sinnott (1914, p. 47) point out: "In determining the possible antiquity of a given character it is essential that its structure, development, and behaviour under different environmental and physiological conditions should be

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[1] The expression "localised stages" has considerable analogy to Goebel's "arrested formations."

studied and compared throughout each representative of a wide range of living and, if possible, of fossil forms." It is all too evident that we have no such complete information in respect to bud scales, a fact which re-emphasises the present danger of theoretical speculation regarding their origin. Goebel (1905, p. 388) assumed that "originally all plants possessed no bud-scales, but arrested or degraded foliage-leaves only appeared as the vigour of vegetation decreased, and that by a very simple process of growth the bud-scales took origin from these arrested forms." Wiegand (1906) made a detailed study of the biological rôle of bud scales and concluded that these structures "have probably been evolved to prevent excessive transpiration and to protect the delicate tissue from mechanical injury." Obviously, however, the phyletic origin of scales cannot be "explained" so easily, for even at the present time, the phylogeny of the foliage leaf and its stipules represent a most complex and obscure problem[1]. As Schrödinger (1919, p. 178) suggested, cataphylls in isolated cases (*e.g. Prunus Laurocerasus* L.) may show a *single* characteristic which goes back to the time of the differentiation of scales from leaves. In general, however, it seems probable that bud scales have evolved along many parallel lines and under several different environments in the past—the details of this evolutionary process may, unfortunately, always remain obscure.

(iv) *The Outlook from a "Causal" Standpoint.*

The evidence which has been critically examined in this paper seems to show clearly that at present we have no satisfactory morphological theory of the bud scale. In several recent papers, Cook (1923 and 1926) has advanced the theory that the appearance of stipular processes on bud scales, sepals and intermediate forms in some cases is an example of "metaphany" or "internal hybridism." In Cook's opinion, such "metaphanic variations" simply represent a re-combination and outward expression of normally latent somatic characters and they may thus possess many "adaptive possibilities" as regards foliar evolution. However, until Cook has furnished cytological and experimental evidence of the re-combination of genetic factors in such organs, his suggestion is of little value.

We have already seen that certain bud scales, *e.g.* the vaginal and stipular "types," may show a more or less close ontogenetic parallelism with the early developmental stages of the foliage leaf—in such cases it may be convenient to regard the "lamina" as an "arrested" structure (cf. also Bower (1916, pp. 703–704), Schüepp (1918, pp. 103–104) and Schrödinger (1914, p. 28 and Pl. III)). In general, however, the conclusion that bud scales are *divergent foliar structures* (as compared with the foliage leaf) appears justifiable, both on the basis of the anatomical evidence advanced by Neese (1916) and Fricke (1926) as well as in the light of comparative ontogeny. The attempt on the part of many investigators to "recover" some of the "formal elements" of the foliage leaf in *every* bud scale or bract is regarded by the writer as a vain and profitless task. Glück's (1919) work in this respect seems

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[1] Cf. Sinnott and Bailey (1914 and 1915). Schiller (1903) interpreted the stipular wings of scales in exstipulate-leaved species as "pseudo-stipules."

a *reductio ad absurdum* for he has recognised no less than four "types" of hypso-phylls, ten "types" of sepals, thirteen "types" of petals and, in the case of stamens, seven "types" of stipular modifications! As Bower (1884, p. 568) pointed out, many years ago, the obvious distinctions which can be drawn between the sheath, petiole and blade of the adult leaf are simply possible as the result of *different types of growth* in the developing organ. Therefore, since the history of development of the bud scale in many instances appears quite *unlike* that of the foliage leaf, neither "formal" nor phylogenetic considerations at present can prove conclusively the existence of "elements" in the scale which are *peculiar* to the mature leaf; this conclusion seems particularly applicable to unsegmented scales and "prophylls." Furthermore, Bower (*op. cit.* pp. 564–571) maintained that Eichler's distinction between the "leaf base" and "upper leaf" rested on the error of considering these parts as co-ordinate and also upon the failure to treat the leaf as a potential branching system. The intercalary growth which separates the leaf base from the frequently branched upper leaf was regarded by Bower as of secondary importance, and he suggested that Eichler's terminology be abandoned. Bower termed the whole main axis of the leaf, *exclusive* of its branches, the *phyllopodium*, which he in turn divided into the hypopodium (*i.e.* Eichler's Blattgrund), the mesopodium (*i.e.* the petiole) and the epipodium[1]. The epipodium designates the upper *unbranched* portion of the leaf axis and is thus only equivalent to Eichler's "Oberblatt" in simple leaved plants. Bower's theory gives us a useful philosophical viewpoint of the "leaf" for it seems unnecessary at present to refer *every* lobe or segment of the bud scale or bract to a stipular appendage of the foliage leaf of some hypothetical ancestral plant. On the contrary, it may be more correct to regard the external conformation of many scales as an example of *homoplasy* rather than of *direct homology*. The factors determining the branching of the "hypopodium" or "epipodium" of the leaf primordium, however, are quite obscure—indeed, save for a few brief remarks by Schüepp (1918), *nothing* seems to be known regarding the location of meristematic tissues in bud scales! As a matter of fact, the available information regarding the history of development of bud scales has been almost entirely based on the *external* form changes of these organs—a study of the mode of internal growth of cataphylls represents a virgin and very promising field for future investigation.

The labour involved in preparing this review will not have been in vain if the writer has shown that at present we are in almost complete darkness regarding two of the most fundamental aspects of the problem of bud-scale morphology, viz. How and under what physiological conditions does the scale develop? The following brief discussion may serve to point out some of the lines of attack which appear promising.

The fact seems well established that in many woody plants the scales and foliage leaves are laid down at distinct and successive intervals during the life-cycle of the plant—the writer suggests that this foliar periodicity may correspond, to some

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[1] Bugnon (1923, 1925 *a*, 1925 *b*, 1925 *c*, 1926 *a*, 1926 *b*) in a recent series of papers, has combined Bower's terminology with the root word "phyllode" in a new set of expressions for the various types of "leaf homologues."

extent at least, with the varying conditions of nutrition at the growing point during organ differentiation. It seems clear from the classical work of Klebs (1903 *a*, 1903 *b*, 1904) that the carbohydrate-nitrogen balance has a most important bearing on the vegetative and reproductive growth of plants. This fact has been repeatedly emphasised by recent investigators, such as Fricke (1926) and André (1927), but at present there are little available quantitative data in regard to the exact relationship of the C/N ratio to foliar differentiation. Whilst the importance of other external and internal factors such as light, temperature and moisture on bud-scale formation has been indicated by the investigations of Goebel (1908) and Klebs (1914), a great deal more information is necessary before we can properly appreciate the intricate physico-chemical changes accompanying morphogenesis (cf. also Dostal (1927)).

In the writer's opinion, two of the most significant distinctions between bud scales and foliage leaves have never received sufficient attention from a "causal" standpoint. Bud scales most frequently are broad, relatively thin structures with a predominately bi-facial type of organisation in contrast to the usually thickened or radially symmetrical base of the foliage leaf. This fact clearly suggests a fundamental difference in the type and localisation of meristematic growth in the scale and leaf—almost no information, however, exists regarding the nature of these growth differences. Apparently in correlation with the characteristic form of bud scales, we find that the internodes between these organs usually remain extremely small and undeveloped in contrast to the situation in the foliage-leaf region of the shoot. Berthold (1904, pp. 208 *et seq*.) has discussed this latter point particularly in regard to the localisation of starch, tannins and sugar in the axis of the winter bud, and it seems likely that further research may show an intimate relationship of the internal metabolism of the growing bud to the formation of bud scales and abbreviated internodes.

Whilst further study of the development and structure of the bud scale *per se* is greatly needed, the fact should be borne in mind that the scale is a periodically formed organ, whose appearance is inseparable from the rhythmical growth and general organisation of the shoot. This viewpoint should help to keep our attention focussed on the dynamic aspects of the problem, the nature and importance of which are only just beginning to be appreciated.

7. ACKNOWLEDGMENTS.

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THE BIOLOGICAL FUNCTIONS OF THE PROTEINS
By DOROTHY JORDAN LLOYD.

WHEN the statement "all flesh is as the grass" was originally made, it summarised the results of philosophical contemplation rather than those of scientific research. The latter, however, though late in the field, have during the last half-century been providing the necessary confirmatory evidence, since every chemical analysis that has ever been recorded of the substance of either plant or animal cells has shown that protoplasm, whatever its origin, is composed of one or more members belonging to the same group of chemical substances. These substances, the proteins, though possessing enormous variety of constitution, have in common certain characteristic atomic groupings which confer on them certain characteristic properties. They are bodies of a colloidal nature and are now known to be formed invariably by the condensation of a number of simpler chemical units, the amino acids. Of these latter substances rather more than twenty have been isolated and obtained in the crystalline state and they form a small but well defined chemical group. The early workers in the chemistry of the proteins, struck by the fact that the same amino acids were frequently isolated from the hydrolytic degradation products of many different animal or vegetable tissues or fluids, believed that there existed a "proteid," a primary living matter, which was a single chemical individual with a highly complex, stable molecule, possessing in itself the properties of assimilation, growth and reproduction. This simple and rather materialistic viewpoint has had to undergo modification in the light of later knowledge. The different proteins are now known to be chemically different individuals, even though all are built up from the same amino acids and conform to the same general plan of structure.

The properties of living matter do not lie in the static equilibrium of a single chemical molecule but in the dynamic equilibrium of a cycle of chemical changes taking place within the limits of the living cell. The most fundamental manifestation of this cycle in both plants and animals is the continuous process of respiration, during which oxygen derived from the atmosphere is used in the combustion of carbohydrates or fats present in the cells, thereby releasing a supply of energy needed for cell synthesis or cell work. In the chemistry of this vital process proteins play no direct part, yet neither respiration nor any other metabolic cycle can take place in their absence. What, therefore, are the biological functions of the proteins? A partial answer to this question is to be found on the one hand in the chemical properties of the amino acids, and on the other hand in those of the great biological solvent, water, which itself, ionising to a slight extent under all conditions with the production of hydrogen and hydroxyl ions, leads to the preponderating

influence in the domain of living matter of all substances having basic or acidic properties. Among organic compounds, the outstanding acidic group is the carboxylic group (—COOH), while the outstanding basic group is the amino group (—NH$_2$). Of all these compounds, therefore, the amino acids, so named since both a carboxylic and an amino group are present in the same molecule, share with water in the highest possible degree the potentiality of releasing either hydrogen or hydroxyl ions into a watery solution. They have also, in common with all other acids or bases, the power of forming salts, which are frequently more soluble than the original acid or base, and which ionise strongly in solution, forming ions which have the capacity of becoming hydrated or associated with water molecules, probably by the orientation round the charged ion of the single molecules or dipoles of the water. They can also form definite compounds with the salts of the alkali metals, which are also probably hydrated in solution. The amino acids, therefore, can, according to the state of their surroundings, display the properties of acids, bases or salts. Hence in aqueous solution they can carry a negative or a positive charge or be iso-electric with the surrounding medium. They can also vary their degree of hydration. They are themselves, however, bodies with small molecules (tryptophane, for instance, which possesses a comparatively complicated nucleus, has a molecular weight of only 190) and hence, when in solution, are freely diffusible across cell membranes. The absolute necessity for a non-diffusible substance as the basis of the living cell cannot be over-emphasised. Colloidal particles are retained by cell membranes. They are, moreover, through the inertia due to the size of the particles, resistant to sudden changes and this inertia or "lag" gives to them an individuality as distinct from the solvent, which is impossible for particles of molecular dimensions in true solution. The proteins, formed by the condensation of a large number of amino acids, possess both amphoteric properties and a structure of colloidal dimensions. They are thus both sensitive and stable, and form, therefore, a suitable basis for the construction of the living cell. By reason of their amphoteric properties they can react with acids or bases to form ionisable salts and by this mechanism they can pass, with changes in the hydrogen ion concentration of the medium in which they find themselves, from the electrically neutral to the electrically charged and from the less soluble to the more soluble (or highly dispersed) condition. In addition, however, by virtue of their colloidal nature, their degree of dispersion is also influenced in neutral, or nearly neutral solutions by the concentration and nature of the diffusible salts present. Dokan (1924) has shown that the degree of dispersion and hydration of the colloidal carbohydrates (agar, konyaku, glycogen) is very largely controlled by these two factors. The same is true of the proteins, especially under conditions where their electric potential towards the surrounding fluids is low. Within the range of pH found in the fluids of the living body, the proteins are particularly sensitive to the influence of salts (Jordan Lloyd and Pleass, 1927). The physical condition of the proteins, therefore, for example their degree of dispersion, extent of free surface, density of charge and degree of hydration, are all under a highly sensitive equilibrium with the acids, salts or bases present in the watery medium in which they

lie. It is impossible to imagine any other organic hydrosol which would be equally sensitive to these conditions. The condition of the proteins in the living cell is directly related to the nature and concentration of the diffusible electrolytes present in the cell or in the surrounding body fluids and as a consequence of this the internal osmotic pressure of the cell, the viscosity of the protoplasm and the distribution of diffusible ions across the cell membrane all react to the stimulus of the cell environment. Is the possession of a highly sensitive physical balance, however, sufficient in itself to account for the omnipresence of proteins in the living cell? And what part, if any, do the proteins of the cells play in the metabolic cycle of energy exchanges? The protein molecule is a large and chemically inert molecule and does not readily enter, as a whole, into a chemical transformation. Whether oxidation of intact proteins proceeds in the cell is a matter for speculation. Under certain limited conditions, intact proteins can absorb oxygen from a gaseous atmosphere (Hopkins, 1925). It is probable, however, that proteins play no *direct* rôle in any cycle of chemical change, without having first been hydrolysed to free amino acids, or at least to peptones, a transformation which takes place without any alteration in the energy values of the system. The direct active agents in the cycle of the living cell are almost certainly small, active molecules. The proteins, with their chemical inertness and high degree of physical sensitiveness, play an overwhelmingly important rôle in the control of the cell equilibrium although they have no direct part in the cycle of chemical changes going on in the cell.

The mechanism by which the proteins exert their influence on cell activities is very simple. They never occur in the cell in the free condition, but always in association, probably frequently as adsorption compounds, with some other cell constituent. Not only do proteins in the cell appear always to be associated in this way, but many of the active cell constituents themselves seem only to exist in association with proteins. This association makes for a high degree of chemical activity associated with an extreme sensitiveness to physico-chemical and physical conditions. Examples of these associated complexes are everywhere abundant in both plant and animal world. The respiratory pigments of animals are compound proteins in which a simple protein is found combined with a comparatively simple chemical individual, the haematin nucleus. These cell constituents are autoxidisable and have spectra which show absorption of light rays in characteristic positions of the spectrum. One of them, cytochrome, occurs widely distributed, not only in animal but also in plant tissues, and appears universally to be combined with protein. It is interesting that in the plant world there is some evidence to suggest that the photosynthetic activity of the pigment chlorophyll, a body closely allied in chemical structure to the respiratory pigments of animals, and to a certain extent similar in physical properties, only carries on its synthetic activity in the presence of (and possibly, therefore, in association with) proteins.

Besides the respiratory pigments, other examples of chemically active systems in which a small active group exists in association with a protein are to be found among the cell enzymes, in the nucleo-proteins which form the chromatin of that very important organ, the cell nucleus and possibly in insulin among the hormones.

THE CHEMISTRY OF THE RESPIRATORY PIGMENTS.

Respiration is, under normal conditions, a combustion of sugar or fat in order to release free energy for cell synthesis. Under conditions of starvation, cell proteins may undergo combustion for this purpose, but this indicates the use of the organism's last reserves. Respiration occurs in plants as well as in animals.

The combustion of the sugar or fat is brought about through the presence in the cell fluids of dissolved oxygen. In multicellular animals the oxygen is brought to the cell by the respiratory pigments of the circulating blood. Of these pigments the haemoglobins found in the erythrocytes of the Vertebrates have been most studied. Haemoglobin is an association of a protein, globin, with an iron-containing porphyrin pigment that readily combines with gaseous oxygen under certain conditions, and as readily gives it up under others. The physiological factors involved in respiration have been worked out largely by Barcroft and his co-workers (see Barcroft, 1924, 1925), and the physico-chemical conditions controlling the gaseous exchange by Henderson and his colleagues (see Henderson, 1926). Both these workers have shown that a proper functioning of the respiratory system depends on the maintenance of the physical conditions of the blood within certain narrow limits. Although the globin of haemoglobin plays no direct part in the automatic oxidation and reduction of the respiratory pigment, it has very definite and important effects on both its physico-chemical and its physiological properties. The crystalline form of pure haemoglobin, and the position of the absorption bands differ in haemoglobin from different species (Reichert and Brown, 1909; Barcroft and Barcroft, 1923). Since there is considerable evidence that the haematin nucleus is the same chemical individual in the haemoglobin obtained from several species, though the globins differ, the specificity of the different haemoglobins has been attributed to their protein moiety (Vlès, 1922; Anson and Mirsky, 1925). The iron-containing haematin nucleus of haemoglobin can be obtained in the free state, or in combination with other nitrogen-containing compounds such as amines or even ammonia. It is only, however, when associated with globin that the pigment is able to show the regular oxidation and reduction changes of respiration under the gaseous potentials found in the living cells. Haemoglobin is a substance widely distributed in the animal kingdom, having been found, not only in the erythrocytes of Vertebrates, but also in the tissue of many of the Invertebrates. Other pigments found in the Invertebrates, such as helicorubin (from the snail, *Helix pomatia*) and actiniohaematin (from certain actiniae), also contain, in combination with proteins, haematin nuclei identical with the haematin nucleus of haemoglobin (Anson and Mirsky). Cytochrome, a respiratory pigment apparently of almost universal distribution in both the plant and animal kingdoms, also contains a haematin nucleus of similar pattern to that found in haemoglobin, again in association with a nitrogenous body, probably a protein (Keilin, 1925). The haemocyanins found in the blood of Arthropods are very similar to haemoglobin, but the nucleus corresponding to haematin contains copper instead of iron. The differences found between the haemocyanins of

different species are undoubtedly due to differences in the globins of the pigments, and not to differences in the nuclei corresponding to haematin (Alsberg and Clark, 1910, 1914). Stedman and Stedman have shown that in pure solutions of the haemocyanins from the lobster and the crab, the pH of the solution affects both viscosity and affinity for oxygen in closely related proportions. Oxygen affinity is greatest where viscosity is least, *i.e.* at the iso-electric point of the protein, and least where viscosity is greatest, *i.e.* under conditions where maximum ionisation may be assumed. This is a clear case in which the condition of the protein influences the functioning of the biologically active group.

Another respiratory pigment, chlorocruorin, a green pigment found in certain Polychaete worms (Fox, 1926), has an iron-containing porphyrin nucleus differing considerably in structure from the haematin nucleus of haemoglobin. The chlorocruorin-haematin nucleus of the pigment is associated with a protein which can be displaced from the complex by amines or ammonia, but here again, as with haemoglobin, the power of automatic respiratory exchange exists only when the protein is present.

The respiratory pigments have been obtained in the crystalline state. A number of simple proteins, for example albumin and the vegetable globulins, are readily obtained as crystals. In these compound proteins the association between protein and pigment is evidently not affected by the building up of the crystal structure and the association may, therefore, be regarded as a true chemical combination, not as an adsorption.

THE CHEMISTRY OF ENZYME ACTION.

Evidence is slowly accumulating that those highly active bodies, the cell enzymes, may also be formed by an association between a protein and a highly active chemical grouping, thus again resulting in a complex which displays both chemical activity and sensitiveness to physical conditions. The only example which has so far been studied in detail is the proteolytic enzyme, peptidase, which has been isolated from macerates of yeast and mammalian pancreas by Fodor and his colleagues (1925, 1926). This enzyme system, the peptidase, consists of a phosphoprotein and an active group, called by Fodor the zymohaptic group. The activity of the enzyme is, however, independent of the presence of protein and the zymohaptic group can be transferred from its association with the protein to an association with kaolin or with glycine. It can also be made to pass into the free state in aqueous solution, but is so unstable in this form that as it becomes free, all activity is immediately lost. The zymohaptic substance itself is diffusible, but it has never been prepared in such a condition that it fails to give certain protein colour tests. Possibly it is of a peptide nature. Fodor considers that under all circumstances the enzyme system consists of a carrier + an active group, the natural carrier in the living organism being the phosphoprotein of the cell. Under these conditions, the activity of the enzyme is controlled by the state of aggregation of the protein and is thus indirectly influenced by hydrogen ion activity. When the active group is transferred from the phosphoprotein on to kaolin or glycine, the

activity is no longer influenced by the hydrogen ion concentration of the medium. The properties of the carrier, therefore, have a very powerful influence on the properties of the enzyme system, and by an association of active group and protein, a system is obtained in which the chemical activity of the zymohaptic substance can be made sensitive to external conditions through the physical sensitiveness of the carrier. Fodor regards the association between the carrier and zymohaptic substance as of the nature of an adsorption and the transference of the zymohaptic substance from substrate to substrate as taking place by elution. The enzyme is most stable when the zymohaptic group is in association with glycine. The nature of this last association cannot be an adsorption in the usual meaning of the term. It is only shown by glycine and leucine among the amino acids tried and seems to suggest that there is no sharp dividing line between association compounds due to residual valencies and adsorption compounds. Fodor considers that in the living cell the zymohaptic group may be transferred from the normal protein carrier to one of the products of the enzyme action, and that it may gain in activity through the transference on to the smaller and more active molecule. Enzyme actions are dynamic processes and the kinetics of enzyme action are chiefly determined by the carriers. When these are proteins, the activity is influenced by the pH value of the system; when they are diffusible peptones, peptides, amino acids or carbohydrates, the activity is independent of the pH of the system.

The work of Fodor and his colleagues on the peptidases of yeast and the pancreas provides an example of the chemistry of enzyme action which has been worked out in considerable detail. The failure which has attended innumerable attempts to free enzyme preparations from protein suggests that the association of physically sensitive protein with a chemically active group may be a general model for all enzyme systems.

THE CHEMISTRY OF THE NUCLEO-PROTEINS.

The nucleo-proteins, which, as their name suggests, are found in the nuclei of cells, are bodies of considerable biological importance. All cell nuclei seem to contain certain nucleo-proteins, though the nucleo-proteins present in cells are not always confined to the nucleus. Nucleo-proteins are complexes consisting of proteins and a well-defined chemical compound, nucleic acid. The association seems to be in the nature of a salt formation between the protein acting as a base and the nucleic acid. From this it follows that only those proteins which have basic properties at the pH value found in living cells (pH 6–8) can exist in this combination and, indeed, so far only the highly basic protamines and histones have definitely been isolated from nucleo-proteins, although there is some reason to suppose that globulins may occasionally function in this manner. Nucleic acid has four ionisable hydrogen atoms, but Hammarsten (1924) has shown that salt formation between nucleic acid and proteins with an iso-electric point of pH 4·7 or greater (such as some globulins and albumins) can only take place at two of these. The more basic proteins can replace all four hydrogen ions. In the nucleo-proteins from bacteria and ripe generative germ cells, the protein seems always to be a

member of the protamines, the most basic sub-group of the proteins. In unripe germ cells the protein associated with the nucleic acid is a histone, a protein less basic than a protamine, but more basic than the albumins and globulins of the cytoplasm. This combination is also found in gland extracts. The nature of the protein in the nucleo-protein of the ordinary somatic cells of plants and animals is not known. References to most of the literature are given by Jones (1920).

Whilst the nature of the protein present in any nucleo-protein has received but little attention from biochemists, the chemistry of the nucleic acid present in the complex has, fortunately, proved more attractive. Nucleic acid is itself a complex molecule, built up by the condensation of four molecules of phosphoric acid, four molecules of sugar and one molecule each of four nitrogenous bases (the purine bases guanine and adenine, and the pyramidine bases cytosine and thymine or uracil). Two types of nucleic acid are known, thymo-nucleic acid, so called because its constitution was first identified in a preparation of nucleic acid made from thymus, and formerly thought to be characteristic of the cell nuclei of animals, and yeast nucleic acid, first identified in a preparation from yeast and formerly thought to be characteristic of plants. The two names are nowadays, however, mainly of historical significance. Thymo-nucleic acid is built on the pattern shown below:

$$
\begin{array}{l}
\text{phosphoric acid} \;—\; \text{hexose} \;—\; \text{guanine} \\
\qquad\qquad\qquad\qquad | \\
\text{phosphoric acid} \;—\; \text{hexose} \quad —\; \text{thymine} \\
\qquad\qquad\qquad\qquad \overset{|}{\underset{|}{\mathrm{o}}} \\
\text{phosphoric acid} \;—\; \text{hexose} \quad —\; \text{cytosine} \\
\qquad\qquad\qquad\qquad | \\
\text{phosphoric acid} \;—\; \text{hexose} \;—\; \text{adenine.}
\end{array}
$$
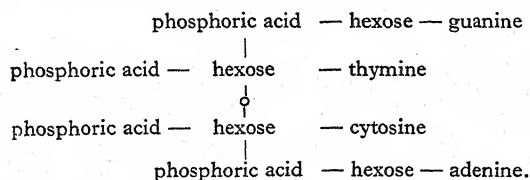
It has been claimed that the thymo-nucleic acids isolated from all animal nuclei are the same substance. Undoubtedly all are built up on the same chemical pattern. Up to the present, however, the hexose groups have not been identified (they are destroyed in the hydrolysis which forms an essential stage in the analysis of the acids) and in bodies which contain four unidentified sugar groupings there is plenty of room for specificity. Levene and Sobotka (1925) have suggested that the sugar in thymo-nucleic acid may be a methylketopentose containing sulphur.

Yeast nucleic acid, obtained from yeast and other plant cells, although constructed on the same general plan as thymo-nucleic acid, shows three striking differences—the carbohydrate group is a pentose, which has been identified as d-ribose, the base, thymine, is replaced by uracil, and the condensation takes place across the four pentose molecules:

$$
\begin{array}{l}
\text{phosphoric acid} \;—\; \text{pentose} \;—\; \text{guanine} \\
\qquad\qquad\qquad\qquad \overset{|}{\underset{|}{\mathrm{o}}} \\
\text{phosphoric acid} \;—\; \text{pentose} \;—\; \text{uracil} \\
\qquad\qquad\qquad\qquad \overset{|}{\underset{|}{\mathrm{o}}} \\
\text{phosphoric acid} \;—\; \text{pentose} \;—\; \text{cytosine} \\
\qquad\qquad\qquad\qquad \overset{|}{\underset{|}{\mathrm{o}}} \\
\text{phosphoric acid} \;—\; \text{pentose} \;—\; \text{adenine.}
\end{array}
$$
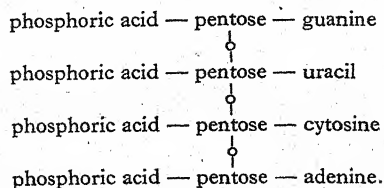
The difference in the structural pattern of thymo-nucleic acid and yeast nucleic acid forms the basis of a method for differentiating these two bodies in plant and

animal cells. A mild acid hydrolysis of thymo-nucleic acid leads to the splitting off of the two bases guanine and adenine, with the appearance in the molecule of the free aldehyde groups of the two corresponding hexoses. Under similar conditions yeast nucleic acid is not hydrolysed. The free aldehyde groups of the hydrolysed thymo-nucleic acid will now give the usual aldehyde reactions, of which the most important is the deep pink colour developed on the addition of fuchsin-sulphurous acid (Schiff's reaction). Feulgen (1924) considers that under carefully controlled conditions, this reaction can be used as a specific test for thymo-nucleic acid in the cell. He has obtained positive reactions in the cell nuclei of the tissues of many animals and higher plants and in ciliate infusoria and he considers that the chromatin of the cell nucleus is in all cases to be identified as thymo-nucleic acid. Breslau and Scremin (1920) obtained positive reactions from the chromatin cf trypanosomes and Robertson (1927) from that of trypanosomes, the flagellate Bodo and many other protozoa. Feulgen obtained negative results to the test with yeast cells. He has put forward the suggestion that yeast nucleic acid may be a body of more primitive type, and that it has been replaced in the higher animals and, to some extent, in higher plants by thymo-nucleic acid. If thymo-nucleic acid is to be identified as chromogen, the special function of yeast nucleic acid needs elucidation. It has been identified in preparations from yeast, plants and fish roes!

Unfortunately chemical studies in nucleic acid have up to the present moment failed to produce a single suggestion as to the function of the cell nuclei in living cells or to correlate in any way the results of chemical investigation with those of biological observation. It is highly probable that in the Mammalia, the normal processes of metabolism involve a continual building up and breaking down of nucleic acids since the urine always contains uric acid or allantoin, both of which have been shown to be derived from the purine bases found in nucleic acid. In man the amount of uric acid in the blood is constant (Denis, 1915) and the amount in the urine depends largely on the amount of purines ingested with the food (Folin, 1905), but even on a purine-free diet there is a continual but small loss of purines in the form of uric acid (Benedict, 1916; Robison, 1922). The Mammalia have undoubtedly a considerable power of synthesising purine bases and the appearance of these in the urine is, therefore, not absolute proof of nuclear katabolism. There is little doubt, however, that the chemical activities involving the nucleo-proteins are mainly connected with the nucleic acid present in them. Nevertheless, in any salt the nature of the base will influence its properties and the nature of the protein partner in the nucleo-protein complex will undoubtedly affect the metabolic cycles in which nucleic acid plays a part.

The correlation between morphological and chemical studies of nuclear activity, the rôle of nucleic acid in the cell and the influence of the nature of the protein base in the chemical activity of the nucleic acids would make a profitable field for research. Dr M. Robertson in a personal communication writes: "Feulgen's reaction is just producing the first opportunity for this kind of research and one very interesting point which I am finding in all this work is the increase in the intensity of the reaction as the diffuse trophic nucleus arranges itself for mitosis.

The bearing of this is still a little obscure and could bear several interpretations. Moreover in the trypanosomes and in Bodo there is present the intriguing para-basal body, a chromatin body of great density lying free in the protoplasm."

THE CHEMISTRY OF THE HORMONE INSULIN.

In the previous section some account has been given of the biological functions of physiologically active complexes formed apparently by the association of a protein and some other group. In the case of the peptidases of yeast (adsorption compounds), and of the respiratory pigments (compound proteins), a definite separation has been obtained between the protein and its associated group. It has also been suggested in recent years that insulin, the active constituent of the internal secretion of the pancreas, consists of an association between a protein and an active group (Langecker and Wiechowski, 1925; Shonle and Waldo, 1925). In this case, however, no separation has ever been accomplished. Abel and his colleagues (1927), who have recently prepared crystalline insulin, state that recrystallisation has no influence on the activity of the compound, and that therefore insulin is not formed by the adsorption of an active body on to a protein, but is a chemical individual of constant composition and molecular structure. Although the latest work of Abel and his colleagues certainly makes it clear that physiologically active insulin differs in this way from say, the physiologically active peptidase of the yeast cell, it still remains possible that insulin may resemble the respiratory pigments and be a body in which an inactive protein may be combined with an active non-protein group.

There is no doubt that insulin contains a protein moiety. It is digested by trypsin (Banting and Best, 1922; Dudley, 1923; Scott, 1925) with a loss of activity proportional to the hydrolytic destruction of the protein constituent (Shonle and Waldo). A more precise classification of the latter into one of the recognised sub-groups has not yet been satisfactorily settled. It contains no phosphorus (Dudley; Scott; Abel and Geiling, 1925), and hence must be excluded from the phospho-proteins. It can withstand boiling for half an hour in centinormal hydrochloric acid (Diujemanse, 1925) and is, therefore, not an albumin or a globulin. In the presence of salt, however, it is irreversibly coagulated by heat at its iso-electric point (Dickens, Dodds, Lawson and MacLagan, 1927). These last workers find that it is completely precipitated from solution by trichloracetic acid and they consider, therefore (following Wasteneys and Borsook, 1923), that it must be either a protein or a metaprotein and cannot be a body of a lower order of complexity such as an albumose, peptone or peptide. Langecker and Wiechowski, however, suggest that insulin may be an albumose. Felix and Waldschmidt-Leitz (1926) believe that it is related to the complex polypeptides, since it is destroyed by pepsin and activated trypsin but not by kinase-free trypsin nor trypsin-free erepsin. Insulin gives the biuret reaction, generally associated with the presence of peptide linkages, and also positive Jaffé and nin-hydrin reactions, usually associated with the presence of diketopiperazine or anhydride rings. Although crude insulin is usually considered non-dialysable (Diujemanse), Dickens, Dodds and co-workers

claim that their purified preparation is slowly but completely dialysable. The nature of the protein half of insulin is, therefore, still under investigation and the work of Best and MacLeod (1923) suggests that it may not be the same in all species. The nature of the active grouping is also still unsettled.

It has already been mentioned above that the only substances so far identified as being condensed into the insulin molecule are amino acids, and it has been suggested on many occasions that the activity of insulin may possibly be attributed to the presence of one particular amino acid which, though condensed into the molecule by a peptide linkage involving either its carboxylic or its amino group, is yet free to exert the chemical activity of some grouping in its nucleus. Out of all the known amino acids the ones which might exert an independent chemical activity are tyrosine, tryptophane, cystine and the more basic amino acids, lysine, arginine and histidine. Of these, tryptophane appears to be absent from insulin, and lysine, though present in small amount, occurs in much larger quantities in crude preparations than in pure preparations of a high activity (Dickens, Dodds and co-workers). These two acids, therefore, vanish from the field of interest. Tyrosine and histidine are both present in insulin, the concentration of the latter increasing with the degree of purity of the insulin (Dickens, Dodds and co-workers). There are, however, no suggestions in the literature that either of these bodies may be the active principle of insulin, and arginine and cystine, therefore, remain to be considered. Cystine contains the chemically active group $R-S-S-R \rightleftarrows 2R-SH$ and arginine, a guanidine nucleus $\begin{smallmatrix} NH \\ NH_2 \end{smallmatrix} \!\!>\!\! C-R$ in which the existence of a second nitrogen atom and a double bond leads to a high degree of chemical activity.

The physiological activity of insulin undoubtedly depends on an oxidation reduction potential, for hydrogen and reducing agents inactivate insulin, while oxygen and oxidising agents restore the activity (Allen and Murlin, 1925; Scott, 1925). It is evidence of this nature, coupled with proof of the existence of labile sulphur in the molecule, that has led du Vigneaud (1927), among other workers, to suggest that the active principle of insulin is the cystine grouping and that this grouping is linked into the molecule by the ordinary peptide linkage. Abel and his co-workers, however, consider that the labile sulphur of insulin only forms 33 per cent. of the total sulphur and that cystine and cysteine are both absent from insulin. In addition, Blatherwick and co-workers find that the content of labile sulphur is not proportional to the activity of a preparation, whilst Sandberg and Brand (1927) point out that certain of the reactions of labile sulphur are also given by arginine, and they suggest that the guanidine grouping of arginine is the seat of the activity. Insulin has indeed a high arginine content, and Frank, Nothman and Wagner (1926) and Minkowski (1926) have prepared synthetic guanidine derivatives which are said to have many of the properties of active insulin.

The physiological activity of insulin is founded on the chemical activity of a small grouping, as yet not identified, but insulin may also exist in the body in an inactive form. There is at present no evidence, however, as to whether this inactivation has been brought about by a reversible chemical device such as a

reduction of the active groups, or by a physical device, such as an alteration of the state of aggregation of the protein as a whole. Insulin seems to lie on the border line between biologically active substances which contain a protein and a chemically active group, and biologically active substances which are peptides. Since the isolation by Hopkins (1921) of glutathione, a new field has been opened on the possible rôle of peptides in animal and plant metabolism.

CONCLUSIONS.

From the review given in the preceding pages of the scanty knowledge available of the part played by the proteins in cell activities, two clearly defined biological functions of the proteins can be deduced. In the first place, the amphoteric and colloidal properties of the proteins make them highly sensitive to changes in composition or condition of the cell fluids and hence establishes a relation not only between the proteins in one cell and those in another but also between these and the external environment of the organism. It must not be overlooked that the inertia of the colloidal particles also gives stability to the system and protects the general equilibrium from sudden localised disturbances. In multicellular organisms sensitiveness to the conditions of the body fluids supplies one mechanism whereby the activities of the different cells or tissues are kept in a harmonious balance throughout the whole organism. Moreover, the living cell draws both the energy and the materials necessary for its existence from the external environment and the power of responding to changes in the external conditions must have been fundamental in the earliest appearance of living matter. The proteins play no part in the metabolic cycles of the living cell, but they exist in the cell associated with chemically active groupings which play a direct part in the cycles of chemical change and the physical condition of the protein affects the chemical activity of the complex.

A second function of the proteins is that they form the chemical basis of the differentiation of species. This possibility of multiplicity of detailed structure combined with uniformity of fundamental structure is due to the large number of amino acid molecules which are condensed together to form the protein molecules. Although only about 20 amino acid species have been isolated from proteins, and although almost every known protein contains almost every known amino acid, yet in bodies which may be formed by the condensation of the order of 100 of the different small units, it is obvious that there is plenty of room for variety.

The chemical individuality of proteins of the same chemical group, but obtained from different species, has been shown many times. Dakin and Dale (1919) showed by an immunological test that the albumins from the blood of hens and ducks, although closely similar in chemical constitution, *i.e.* yielding the same amino acids in very similar proportions, were actually different proteins in the two species, and Dudley and Woodman (1915) showed that the casein from sheep's milk is not the same substance as the casein from cow's milk. Not only the nature and relative proportion of the different amino acids, but also the actual order of arrangement in the molecule, influence the physical properties of the protein (Dakin and Dale);

even the opening of ring structures or the closing of open chains, or keto-enolic transformations in the molecule are accompanied by a change in physical properties. The chemistry of the proteins therefore offers a reasonable explanation of the undoubted fact that although many biochemical reactions are common to a great number of different organisms, yet "protoplasm" is undoubtedly different in each species.

In sharp contrast to the biologically specific character of the different proteins, it can be seen that the biological distribution of chemically active cell constituents is frequently very wide. Glutathione, for instance, occurs in yeast, proliferating plant tissues, and all metabolically active animal tissues. Insulin, or bodies with insulin-like properties, has been isolated from the pancreas of mammals and fish, and from yeast. Cytochrome, with a haematin nucleus which appears to be the same chemical individual in all species, has a distribution as wide as, or even wider than, glutathione. Judging again by their action on different substrates, the distribution of the active principle of an enzyme is not limited to one species but is very wide. Quastel (1926) has put forward the thesis that the dehydrogenases of *B. coli* are the same enzyme, the activities of which are controlled by the oxidation-reduction potential of the cell. His arguments may apply with equal force to the dehydrogenases of different species.

Not only in the cytoplasm of the cell but even in the cell nuclei, the active chemical groups seem to be non-specific in character. Thymonucleic acid seems to be the same chemical individual whether it is isolated from the sperm cells of fish, the thymus of mammals or even as it exists in the Protozoa or the higher plants.

It must be emphasised that there is no wish to suggest that the chemical cycles found in the metabolism of any one plant or animal species necessarily exist throughout the whole of the animal and vegetable kingdoms. There is plenty of evidence to show that in the course of evolution many different means have appeared to serve the same end. The existence and distribution of the different respiratory pigments, haemoglobin, haemocyanin, chlorocruorin, etc., shows that in the animal world, a number of respiratory pigments have been evolved independently and at different times. Further, in the plant world, proteins only occur as intra-cellular substances or as food reserves in seeds, while in the animal world they have in addition been used by the organism to build up extra-cellular tissues, such as the protective keratins of the epidermis and its accessory structures or the supporting fibres of the connective tissues. In plants, protective and supporting tissues are always built up from carbohydrates and this contrast alone between plants and animals show that evolution has a chemical basis. It is certain, however, that a metabolic cycle, once it is established as part of the dynamic equilibrium of living protoplasm, may persist, unchanged in its essential features throughout the differentiation of a very large number of species. The chemically active groups such as the haematin nucleus of the haemoglobins, the zymohaptic groups of enzymes, the nucleic acids of the nucleoproteins are all chemical individuals with small molecules and unstable energy content. Through their association with the cell proteins in colloidal complexes, highly sensitive to physical conditions, their activities in the cells of any

organism are controlled by the state and composition of the fluids of the cell and thus indirectly come under the influence of the external environment. The hydrogen ion activity and concentration of salts in the cell fluids, for instance, are influenced by the environment and in their turn affect the state and activity of the protein complexes.

Through the slightly different reactions of the different individual proteins to the same external stimulus, the specific cell proteins thus become also the basis of the specific variations found in the metabolic cycles of living organisms.

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LES DONNÉES EXPÉRIMENTALES RELATIVES AU MÉCANISME DE LA DIVISION CELLULAIRE

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(Chargé de Cours à l'Université de Bruxelles.)

(Received March 12th, 1928.)

(With Fourteen Text-figures.)

MALGRÉ les énormes efforts déjà consacrés à l'étude de la division cellulaire, les biologistes ne cessent de s'intéresser à ce problème fondamental. A côté de nombreux travaux originaux, ces deux dernières années ont vu paraître un livre fort attachant de A. Gurwitsch (1926), envisageant le côté physiologique de la division cellulaire, tandis que Prat et Malkovsky (1927) publiaient une mise au point très complète des causes de la croissance et de la division cellulaire. Cependant, ces auteurs n'ont guère considéré le mécanisme intime de la cytodiérèse—au sens large de ce mot. Ils ont eu surtout en vue ce processus en tant que réaction à des influences extérieures à la cellule, que celles-ci se trouvent dans le milieu ou dans l'organisme lui-même.

C'est un point de vue de la plus haute importance, mais les notions qui s'y rattachent perdent un peu de leur valeur si l'on ne s'efforce pas d'analyser parallèlement les évènements intrinsèques de la mitose ou de l'amitose. Or, nous possédons à ce sujet un ensemble déjà considérable de renseignements. Les uns ont été tirés de l'observation patiente et minutieuse de la cytodiérèse normale, en ayant recours dans de nombreux cas aux ressources de l'investigation expérimentale. Les autres découlent de diverses expériences dans lesquelles on a réussi à modifier le cours naturel des phénomènes, en suscitant des "anomalies" variées de la division cellulaire. Ce sont surtout ces modifications expérimentales que je crois utile de rassembler ici. Non pas que je leur attribue une signification plus grande qu'aux données de l'observation, bien au contraire. Qu'il s'agisse de l'étude purement cytologique et descriptive, ou de ses compléments modernes qui nous éclairent sur la consistance, la viscosité, la perméabilité, les courants internes, bref les propriétés physiques et physiologiques du cytoplasme, l'observation de la cellule normale doit rester à la base de toute interprétation. Mais les notions qui s'y rattachent ont vite acquis droit de cité dans les ouvrages classiques, les essais d'interprétation générale en tiennent suffisamment compte et je pourrai me borner, ici, en ce qui les concerne, à de brèves allusions. Les modifications expérimentales de la cytodiérèse semblent moins connues et surtout moins appréciées; beaucoup ont été recueillies incidemment au cours de recherches d'ensemble; éparpillées dans des mémoires dont les uns sont déjà assez anciens, les autres tout récents, elles échappent aisément à l'attention. De plus, nombre de cytologistes conservent une sorte de prévention

contre ces "anomalies," d'autant plus que la plupart d'entre elles vouent la cellule à une déchéance prochaine. C'est un tort, cependant, que de les considérer sous cet angle péjoratif. Même abortives, les anomalies de la division cellulaire sont notre seule ressource pour dissocier les rouages complexes de ce processus. Leur valeur est d'autant plus certaine qu'elles reproduisent souvent des modalités rencontrées dans l'évolution normale d'autres espèces. La faculté de les faire surgir à volonté par des facteurs aisément maniables, permettra sans doute d'en creuser davantage le déterminisme. En un mot, dans ce domaine comme dans celui de la morphogénèse, la distinction entre les potentialités réelles et totales garde son incomparable valeur. Nous ne pourrons nous forger une idée exacte et complète de la division que si nous savons non seulement ce que fait la cellule à cette phase critique de son existence mais encore tout ce qu'elle peut faire. C'est la condition préalable de l'édification d'une théorie de la physiologie de la division cellulaire, dont il n'existe encore, malgré de louables tentatives, que des linéaments fragiles.

La question que nous nous posons ici est donc la suivante: dans quels cas, par quels moyens et dans quelle mesure pouvons-nous actuellement modifier le cours de la division cellulaire? A défaut de tout résultat concernant l'amitose, nous n'aurons à traiter que de la division indirecte, et il sera préférable, pour éviter toute confusion, que nous nous limitions aux seuls cas d'action immédiate sur la cellule. J'entends surtout, par cette restriction, écarter le problème tout différent de la croissance cellulaire, et par le fait même les innombrables circonstances où nous suscitons ou arrêtons des proliférations cellulaires par une action certaine ou possible sur la croissance. Citons par exemple l'effet de la thyroïde sur la métamorphose, ou encore celui des poisons caryoclasiques bien étudiés par Dustin, la réaction de caryocinétose de Paillot, l'induction mitogénique de Gurwitsch, les facteurs si complexes de prolifération des cultures de tissus, etc. Dans tous ces cas, pour lesquels je renvoie à l'article de Prat et Malkovsky, il est impossible de discriminer avec certitude si nous agissons sur la croissance ou si, au contraire, nous provoquons ou empêchons la division des cellules à trophisme constant. Il n'y a guère qu'un matériel où la dissociation entre la croissance et la division soit formellement réalisée, c'est l'œuf des métazoaires, où la phase cinétique du "métabolisme constructif" ne survient qu'après la phase de "métabolisme accumulatif" qui est la période de grand accroissement de l'oocyte. Aussi la majorité des modifications expérimentales de la cytodiérèse concerne-t-elle les cinèses des œufs vierges ou fécondés. Les longues recherches qu'ont suscitées la fécondation et la parthénogénèse ont permis de glaner des "anomalies" étrangement variées. Et il paraît possible, comme j'espère le montrer ici, de les grouper de façon à dégager les corrélations que présentent des images à première vue hétéroclites.

LA PROPHASE MITOTIQUE.

Quel est le premier indice annonciateur d'une mitose? Est-ce un remaniement des particules de caryotine, un début d'ordonnancement en chromosomes visibles? Est-ce l'apparition d'une irradiation cytoplasmique, ou la division du centrosome, ou encore le gonflement prophasique du noyau? Cela varie certainement selon les

espèces et les types cellulaires, mais il est constant que des modifications surgissent soit dans le noyau soit dans le cytoplasme avant que la dissolution éventuelle de la membrane nucléaire ne confonde ces phases distinctes de la cellule au repos.

Ces processus initiaux sont parmi ceux qui échappent le plus à notre contrôle. Mais nous commençons cependant à discerner les causes de la réapparition des chromosomes. Les patientes études de T. Sakamura (1920, 1927) sur les cellules végétales ont mis en évidence l'effet d'un abaissement du pH[1]. En faisant baigner les cellules-mères du pollen de *Tradescantia virginica*, de *Lilium*, de *Paris quadri-folia* dans une atmosphère de CO_2 ou en les soumettant à divers réactifs—parmi lesquels divers sels inorganiques—ce botaniste a pu faire apparaître les chromosomes dans les noyaux quiescents. Ce changement semble consister en une condensation des parcelles de caryotine réparties dans le territoire de la caryolymphe répondant à chaque chromosome. Sa condition majeure paraît résider en une augmentation de la concentration des ions H^+, mais sans doute n'agit-elle pas seule car l'auteur a obtenu avec divers sels des résultats positifs, mais pas toujours cohérents. L'in-dication n'en est pas moins intéressante. Il s'agit là de réactions extrêmement délicates, d'autant plus que le degré de maturité du noyau intervient probablement. On sait que dans les mêmes cellules-mères du pollen de *Tradescantia* Chambers et Sands (1928) ont vu se dessiner les chromosomes grâce à la simple pénétration de l'aiguille à microdissection, mais cela uniquement dans les cellules qui étaient sur le point de se diviser. Nous touchons d'ailleurs là au problème épineux du com-portement des chromosomes au cours de la prophase des divisions réductionnelles. Sans y voir plus qu'un rapprochement, il est intéressant de noter avec Runnström que des images d'appariement ont été obtenues dans des cellules somatiques soit chez les plantes (Nemec, 1906)[2] soit chez les animaux (Häcker, 1900; Schiller, 1909). Runnström lui-même a vu surgir des aspects réductionnels dans des œufs fécondés d'oursin cultivés dans une eau de mer privée de K. Et puisque nous rencontrons ici cette question de la relation entre la morphologie des chromosomes et la composition du milieu, mentionnons que chez *Asterias glacialis* la culture des oocytes vierges dans une eau de mer diluée fait régulièrement apparaître dans la 1e figure de maturation (qui présente une polycentrie dont il sera question plus loin) un chromosome de volume considérable (Fig. 1). Cette observation a son intérêt comme témoignage des différences constitutives entre les chromosomes d'un même assortiment (Dalcq, 1923).

L'apparition d'une gélification centrée dans le cytoplasme est également un phé-nomène que nous pouvons provoquer depuis la découverte par T. H. Morgan (1895) des asters accessoires que peut faire surgir, selon les cas, l'action de certains sels, la narcose, l'agitation mécanique. Mais la question du rôle cinétique de ces néofor-mations est assez compliquée et nous l'envisagerons plus loin à propos de la dicentrie.

Examinons auparavant le processus de dissolution ou de désagrégation de la membrane nucléaire. Cet évènement n'est pas absolument constant dans la division indirecte. Il fait défaut chez de nombreux Protistes, et l'on rencontre incidemment

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[1] Voir aussi Kuwada et Sakamura (1927).
[2] Lundegårdh (1914), Sakamura (1920) se sont élevés contre l'interprétation de Nemec.

13-2

chez les animaux des exemples de figure mitotique intranucléaire, dont celui qu'a figuré Hegner (1908) chez *Camptocamptus* est un des plus typiques. Cela ne signifie
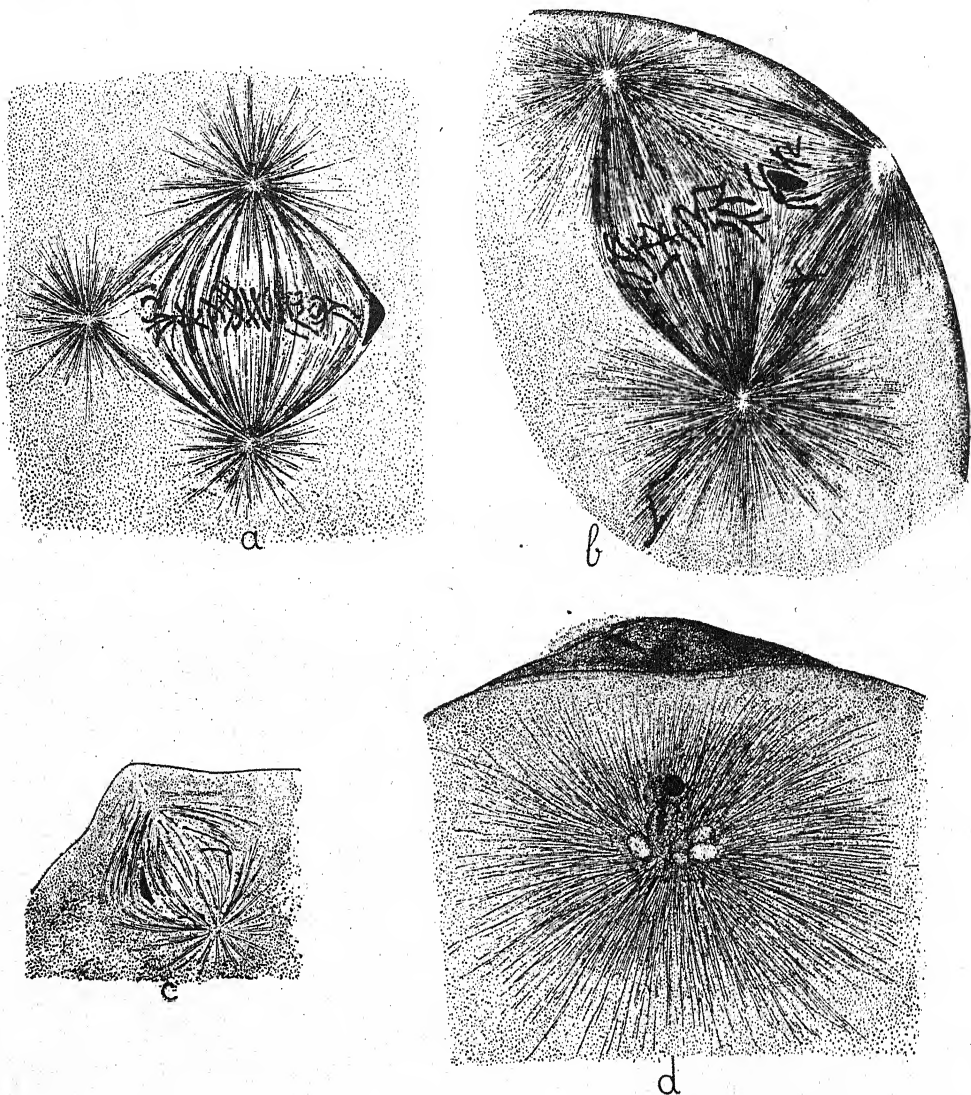
Fig. 1. Œufs vierges d'*Asterias glacialis* cultivés dans eau de mer 55 c.c. + H₂O 45 c.c. *a*, métaphase de 1er ordre tripolaire; *b*, début de l'anaphase; *c*, isolement d'un globule polaire géant; *d*, reconstitution des caryomères. Dans chaque mitose, le chromosome volumineux apparu sous l'influence de la dilution du milieu.

évidemment pas qu'en pareil cas le noyau évolue d'une façon indépendante du cytoplasme. On conçoit aisément que la membrane nucléaire puisse laisser filtrer dans le noyau bien des substances. L'éventualité opposée est d'ailleurs tout aussi possible et les longues discussions auxquelles on s'est livré pour savoir si l'aster

était lors de son apparition intra- ou extranucléaire n'ont, du point de vue fonctionnel, qu'un intérêt minime. Ces localisations dépendent simplement du taux et du sens de la diffusion ou de la perméation à travers la membrane nucléaire, et en attendant des informations plus précises, l'attitude la plus impartiale est d'admettre que les évènements annonciateurs de la division indirecte, gélification astérienne et condensation des chromosomes, dépendent de l'interaction cyto-nucléaire.

D'une manière générale, la modification de la membrane nucléaire ne se borne pas à une perméation discrète, mais va jusqu'au ramollissement et à la dislocation; elle entraîne ainsi le mélange au moins apparent du matériel nucléaire avec le cytoplasme. Dans le cas des oocytes de divers Invertébrés, on voit nettement par la chronologie et la localisation des évènements que la membrane nucléaire perd d'abord sa turgescence (signe de perméation), puis se ramollit en un point, souvent rapproché du pôle animal; une gélification radiée[1] y apparaît instantanément, tandis que, de proche en proche, la membrane se disloque et libère le contenu nucléaire. Et comme l'intervention de certaines conditions de milieu suffit à déclencher cette entrée en maturation, il est légitime de penser que sa cause immédiate réside dans une modification du cytoplasme qui affecte la membrane nucléaire. Faisons ici abstraction, pour les espèces où elle se présente, de l'action naturelle de la fécondation sur l'entrée en maturation et confrontons les cas où l'on a pu, jusqu'à présent, provoquer artificiellement ce phénomène dans l'œuf vierge. Ce sont:

1º. *Asterias forbesii.* Loeb (1902) avait noté que dans les pontes rebelles à la maturation spontanée dans l'eau de mer l'alcalinisation favorise la disparition des vésicules germinatives. Miss M. Brailey a publié en 1923 des observations sur les œufs vierges de la même espèce, mais cet auteur n'a pas établi la distinction pourtant évidente entre l'entrée en maturation et l'expulsion des globules polaires. Son mémoire examine très complètement les conditions nécessaires ou favorables à l'expulsion de deux globules polaires normaux, mais ne signale qu'incidemment que la vésicule germinative peut se flétrir dans l'eau de mer additionnée de KCN.

2º. *Asterias glacialis.* En 1924, j'ai montré que, dans des conditions égales de pH et de pression osmotique, des quatre principaux chlorures de l'eau de mer le $CaCl_2$ est le plus favorable à la rupture de la vésicule germinative et que, dans les pontes "immatures" et par conséquent rebelles à la maturation spontanée dans l'eau de mer, le $CaCl_2$ augmente la proportion des entrées en maturation. L'immersion d'un ovaire entier dans le $CaCl_2$ permet d'obtenir le flétrissement de la vésicule germinative dans les oocytes contenus dans la glande, pourvu qu'ils soient parvenus au terme de leur accroissement.

3º. *Sabellaria.* L'oocyte vierge de cette espèce flétrit en général sa vésicule germinative lorsqu'il parvient dans l'eau de mer mais ne poursuit son évolution autonome que jusqu'à la métaphase de la 1e mitose de maturation. Fauré-Frémiet (1921) a constaté que cette "prématuration" est favorisée par une légère alcalinisation et qu'elle est entièrement inhibée lorsque le pH descend à 5,6. Elle se produit en présence de KCN ainsi que dans l'eau de mer privée d'oxygène.

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[1] Chez le chætoptère, l'oursin, l'astérie, cette gélification est d'abord polycentrique.

4°. *Pomatoceros.* Chez cette espèce voisine de la précédente, Hörstadius (1923) observe également un effet favorable de l'alcalinisation. Mais il constate, en outre, en dehors de toute variation du pH et de la pression osmotique, une action caractéristique du KCl et du CaCl$_2$. Si à une eau de mer artificielle (formule de Meyerhof) sans KCl on ajoute progressivement ce sel, la proportion d'entrées en maturation s'abaisse rapidement. Si au contraire on part d'une eau de mer artificielle sans CaCl$_2$, l'adjonction progressive de ce sel entraîne pour les doses faibles une augmentation des maturations; mais bientôt un maximum ·est atteint et la proportion des maturations s'abaisse jusqu'à une inertie presque totale (Fig. 2).
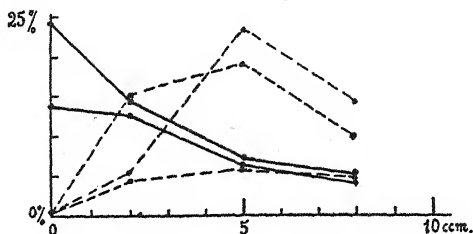
Fig. 2. Percentage de maturations de l'œuf vierge de *Pomatoceros* en fonction de la composition du milieu. *En trait plein*, effet de l'addition de KCl isotonique à l'eau de mer artificielle de Meyerhof sans KCl. *En pointillés*, effet de l'addition de CaCl$_2$ à l'eau de mer artificielle sans CaCl$_2$. D'après Hörstadius.

5°. *Lottia gigantea.* Chez cette patelle, Loeb (1906) paraît avoir obtenu la maturation d'une part par l'addition d'un alcali à l'eau de mer, d'autre part grâce à un traitement par l'eau de mer additionnée de benzol, puis retour au milieu naturel. Il est cependant difficile de déduire du texte si la maturation s'entend bien ici au sens habituel ou dans celui d'aptitude à la fécondation. Il n'est nulle part question de vésicule germinative ni de globules polaires, alors qu'on rencontre l'expression "eggs are induced to mature."

6°. *Mactra.* Dans ses recherches bien connues sur la parthénogénèse de la Mactre, Kostanecki (1904) a utilisé l'eau de mer additionnée de KCl hypertonique. L'œuf étant pondu à l'état d'oocyte de 1er ordre, le premier effet de cet enrichissement en K a donc été la rupture de la vésicule germinative. Mais il est clair qu'une analyse plus approfondie serait nécessaire pour nous faire connaître la cause exacte de cette entrée en maturation.

7°. *Barnea candida.* Ce Lamellibranche des côtes du Boulonnais ne présente jamais de maturation intraovarienne. Les oocytes de 1er ordre sont pondus munis de leur vésicule germinative et c'est normalement la fécondation qui détermine le flétrissement du noyau. Mais ce processus peut se produire aussi dans l'œuf vierge et cela, comme je l'ai constaté l'an dernier, sous deux influences. La première consiste dans l'addition d'un peu d'alcali à l'eau de mer; l'alcalinisation d'une solution pure de NaCl, à laquelle ces œufs résistent cependant parfaitement, ne détermine la disparition de la vésicule germinative qu'au seuil de la cytolyse. Bien que l'analyse expérimentale présente à ce point de vue des lacunes, il y a lieu de croire que l'alcalinisation n'agit qu'en fonction de la présence de certains sels dont l'activité est utilisée dans le second procédé. Celui-ci consiste dans l'emploi, à pression osmotique et pH égaux, du CaCl$_2$ et du KCl. On démontre l'efficacité de ces agents en recueillant les œufs au sortir de l'ovaire, sans contact avec l'eau de mer, dans un mélange dit inactif où entrent NaCl, MgCl$_2$, MgSO$_4$, CO$_3$HNa

dans les proportions de la formule de Meyerhof. On répartit alors la culture en des lots homogènes que l'on enrichit à des degrés différents soit en $CaCl_2$, soit en KCl ou en $CaCl_2$ et KCl à la fois. Naturellement, le degré de maturité intrinsèque de chaque ponte introduit dans les résultats une variété inévitable. Mais quelle que soit la ponte utilisée, toujours on peut obtenir l'entrée en maturation de tous ses oocytes accomplis en introduisant dans le milieu une quantité suffisante de KCl et de $CaCl_2$. La Fig. 3 en montre un exemple. Les œufs d'une ponte ont été
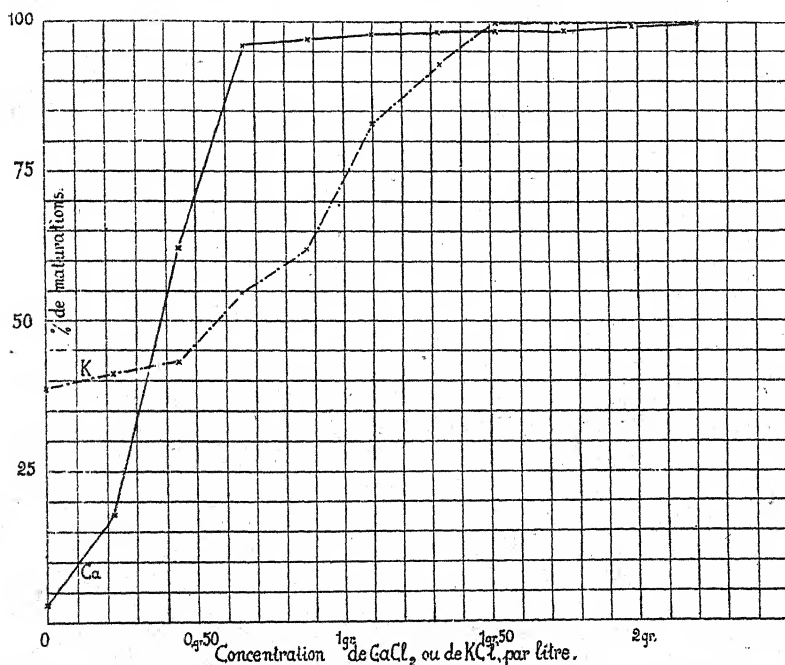
Fig. 3. Effet réciproque du Ca et du K sur la maturation de l'oocyte vierge de *Barnea candida*. *Trait plein*: œufs pondus dans un mélange inactif + KCl (2 °/₀₀) et additionnés secondairement de $CaCl_2$ aux concentrations indiquées en abscisses. *Trait interrompu*: œufs de *la même femelle* pondus dans un mélange inactif + $CaCl_2$ (2 °/₀₀) et additionnés secondairement de KCl.

recueillis dans le mélange inactif, puis partagés en deux cultures. La première a été additionnée de 5 c.c. de $CaCl_2$[1], puis répartie en lots homogènes auxquels du KCl a été ajouté à doses progressives. Vice-versa, la seconde culture a été saturée par 5 c.c. de KCl[1], puis répartie en lots homogènes auxquels du $CaCl_2$ a été ajouté à doses progressives. Après un temps suffisant pour que la réaction ait atteint dans tous les cristallisoirs son état d'équilibre, j'ai pratiqué le dénombrement des œufs inertes et des œufs en maturation dans chacun des lots. On voit que dans les deux cas la proportion des maturations s'accroît avec une remarquable régularité dans les deux séries de cultures. Son ascension n'est pas linéaire mais affecte une allure en S, qui se retrouve dans tous les tracés. En analysant cette courbe, dont l'allure est due à la légère hétérogénéité physiologique de la "population" oocytaire

[1] Isotonique et de même pH que l'eau de mer.

sur laquelle porte l'expérience, on arrive à dégager cette notion que l'entrée en maturation est une réaction mitotique douée d'un seuil chimique précis et qui représente, à l'échelle cellulaire, une application de la grande loi du tout ou rien de Pézard. Pour des raisons de détail que j'ai développées ailleurs (1928), il est probable que le rôle du Ca dépasse celui du K, qui n'est peut-être qu'un agent de perméabilisation spécifique des oocytes à l'égard de l'autre cation.

Si nous jetons maintenant un coup d'œil d'ensemble sur le déterminisme du flétrissement de la vésicule germinative, nous apercevons dans ce processus un déterminisme uniforme chez des espèces appartenant aux groupes relativement éloignés des Échinodermes, des Vers, des Mollusques. Malgré la diversité des techniques, en partie imposée par l'organisation des formes étudiées, nous distinguons clairement deux agents essentiels de maturation : 1°, les ions OH (*Asterias, Sabellaria, Lottia, Pomatoceros, Barnea*) ; 2°, l'ion Ca (*Asterias, Pomatoceros, Barnea*), éventuellement avec intervention adjuvante du K. Mais peut-être l'unité est-elle plus grande encore, car les expériences d'alcalinisation ont presque toutes été réalisées en présence de l'eau de mer totale, et il est possible que l'élévation du pH, dont l'action perméabilisante aux sels est bien connue, ne fasse que faciliter la pénétration des sels spécifiquement efficaces.

L'ÉDIFICE MITOTIQUE.

D'une manière générale l'édification de la figure mitotique normale s'accomplit chez les Métazoaires et les Métaphytes par deux processus distincts au point de vue cytologique. Chez un grand nombre de cellules, il existe à l'état de repos une sphérule centrosomiale distincte, de structure plus ou moins compliquée, souvent pourvue de deux centrioles. A l'approche de la division, c'est autour de cette sphérule que se dessine l'irradiation qui subit bientôt, suivant l'axe passant par les centrioles éventuels, un allongement qui rend évidente la dicentrie. Avec des modalités variables, le fuseau se constitue peu à peu entre les pôles et le matériel nucléaire s'intègre à l'édifice achromatique. Dans un nombre de cas plus restreint, mais cependant appréciable, il n'existe pas de centrosome décelable. Le fuseau et, si elles existent, les irradiations polaires, se développent de façon en apparence spontanée, sans que l'on distingue d'organite dont procèdent ces structures achromatiques.

Or, on n'a guère eu jusqu'à présent l'occasion d'expérimenter sur des cellules évoluant suivant le type à centrosome différencié. A ce stade encore de la mitose, ce sont surtout les œufs en maturation ou en segmentation dont on a pu influencer l'activité, et précisément dans les formes utilisées, il n'existe pas de granule centrosomial constamment perceptible sauf en ce qui concerne le centrosome spermatique, dont il semble bien, pour des raisons que je toucherai plus loin, que l'on ait surestimé l'importance.

C'est pourquoi, dans l'exposé des modifications expérimentales de la figure mitotique, j'envisagerai en première analyse les centrosomes et spécialement les pôles mitotiques comme des états fonctionnels du cytoplasme de la cellule en division. En d'autres termes plutôt que d'accorder à priori aux astrophères la

propriété intrinsèque de se diviser, suivant la conception classique de Boveri, je m'efforcerai de distinguer la nature des conditions extrinsèques aux centrosomes et capables de déterminer leur subdivision, celles qui seraient, par conséquent, l'agent de la dicentrie si caractéristique de la mitose. J'examinerai ensuite comment l'opinion à laquelle conduit ce point de vue est conciliable avec la présence et le rôle apparent du centrosome figuré.

Ce qui justifie surtout cette initiative, c'est qu'il est aujourd'hui acquis que les asters accessoires, surgis de novo dans le cytoplasme en l'absence de tout granule
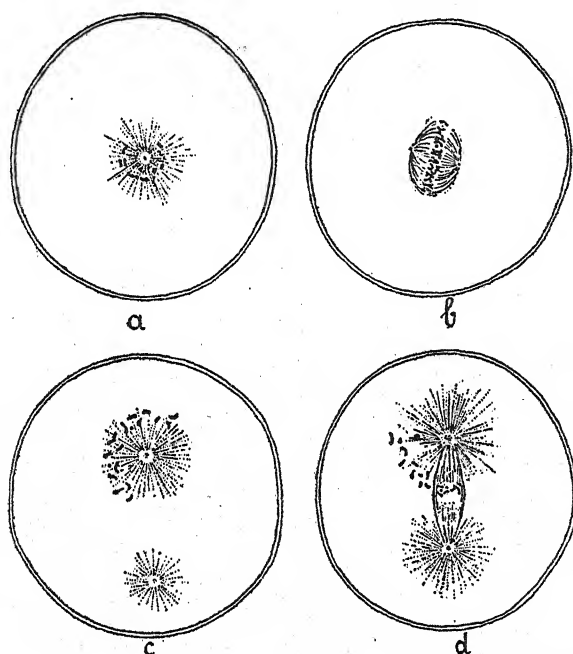
Fig. 4. Œufs vierges d'oursin traités par la méthode de Loeb. *a* et *b*, œufs soumis simplement au premier temps (traitement butyrique); *a*, simple monaster; *b*, fuseau anastral en tonnelet; *c* et *d*, œufs soumis en outre au second temps; *c*, apparition d'un aster accessoire à proximité du monaster; *d*, formation d'un amphiaster par connexion du monaster et du cytaster. D'après Herlant.

préformé, peuvent réellement avoir la même valeur fonctionnelle que les asters principaux. En effet, ce qui caractérise ceux-ci, au point de vue physiologique, c'est leur rôle en tant que pôles mitotiques, leur faculté au moins apparente d'attirer les chromosomes anaphasiques, le pouvoir, spontané ou non, de se diviser pour former les pôles de la cinèse suivante. Ces attributs, un aster accessoire peut les posséder. Herlant (1919) a montré que dans certains œufs vierges d'oursin le traitement par le procédé classique de Loeb fait apparaître côte à côte un monaster ovulaire et un aster accessoire. D'abord indépendantes, ces deux formations peuvent ensuite se mettre en rapport par un fuseau sur lequel viennent se disposer des chromosomes (Fig. 4) et l'achèvement de cette mitose s'accompagne de plasmodiérèse. Fry (1925) a vérifié ce fait et constate que le clivage se poursuit au delà du stade II.

Toutefois, il faut se garder de conclure de cette donnée expérimentale à l'équivalence fonctionnelle absolue des asters accessoires et principaux. En réalité, il s'agit là d'un problème assez délicat, que nous poserons clairement en énonçant les principales modifications expérimentales que l'on a fait subir à la mitose de segmentation: Boveri (1896) a constaté sur des œufs d'oursin soumis à l'agitation mécanique, que les chromosomes pouvaient accidentellement passer tous à l'un des pôles, et que le clivage du blastomère achromosomial ne s'en poursuivait pas moins. Ziegler (1898) a fait la même observation sur des œufs d'oursin soumis à une légère compression. Certes, comme le remarque Fry, la technique employée dans ces deux cas ne donne pas la certitude que l'un des pôles soit absolument privé de chromosomes, mais la présomption est néanmoins très forte.

McClendon (1907) s'est adressé à des œufs vierges d'astérie. Pendant leur maturation, il a enlevé à l'aide d'une fine pipette la région du pôle animal où pointait le globule polaire et a soumis les œufs ainsi privés de chromosomes à l'activation par le CO_2, suivant la méthode de Delage. Dans un certain nombre de cas, il a obtenu la segmentation jusqu'à un stade avancé (Fig. 5) et l'examen cytologique n'a pas révélé trace de chromosomes. Fry (1925) a sectionné au scalpel des œufs d'oursin de façon que le pronucleus femelle soit compris tout entier dans un des fragments, puis a traité les deux parties de chaque œuf par l'acide butyrique et le NaCl hypertonique, conformément à la technique de Loeb. Par cette étude individuelle, il a vu que si la portion anucléée forme des asters accessoires parfois nombreux, ceux-ci ne présentent que des phases cycliques d'expansion et de régression, mais jamais ils ne se divisent.

Fig. 5. Œuf vierge d'astérie dont le fuseau de maturation a été enlevé, puis qui a été soumis à l'activation par le CO_2. Chaque blastomère contient un ou plusieurs cytasters, mais point de noyau ni de chromosomes. D'après McClendon.

Au contraire, dans la portion nucléée ou dans l'œuf entier, il est fréquent que des asters accessoires se divisent, mais ce sont uniquement ceux qui, en raison de leur situation, ont pu capter un certain nombre de chromosomes (Fig. 6). D'où la conclusion tirée par l'auteur américain, que les chromosomes exercent une action de présence sur la division des astrophères.

Si l'on se soucie d'aller au fond des choses, ces trois constatations expérimentales ne sont pas aisément conciliables. D'une part, qu'ils soient principaux ou accessoires, des asters sans chromosomes poursuivent leur évolution, engendrent un fuseau, suscitent la plasmodiérèse, d'autre part des asters accessoires paraissent, en l'absence de chromosomes, incapables d'activité cinétique. Certes, la technique de Fry est de loin la meilleure et l'on serait tenté, comme il le suggère, de douter de la réalité de l'élimination des chromosomes dans les expériences de Ziegler, Boveri, McClendon. Ce jugement paraît cependant trop radical, surtout au point

de vue de l'expérience de McClendon et l'on éprouve d'autre part quelque peine à admettre la conclusion de Fry si l'on considère le cas des œufs à monaster. Que ceux-ci soient obtenus par le premier temps de la méthode de Loeb ou par la fécondation sans pénétration du spermatozoïde, processus dont on connaît aujourd'hui de nombreux exemples[1], on y trouve un véritable aster principal nanti de chromosomes; la plage centrale de cette centrosphère souvent énorme reste généralement globuleuse, parfois elle s'étire légèrement comme on le voit dans une figure de Ziegler (1898), d'autre fois encore elle est remplacée par un fuseau trapu, anastral, comme Bataillon l'a signalé à diverses reprises dans les fécondations
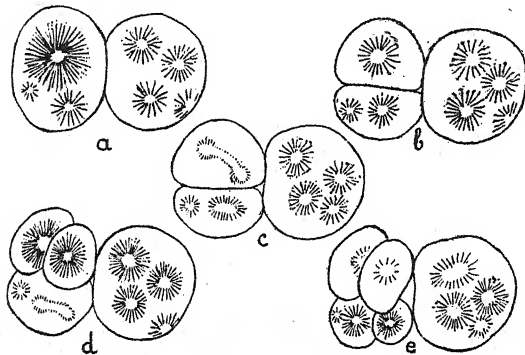
Fig. 6. Œuf vierge de scutelle sectionné dans sa gangue, puis soumis aux deux temps de la méthode de Loeb. A gauche le fragment nucléé, capable de segmentation, à droite le fragment anucléé où quatre cytasters sont apparus et restent indivis; *a*, après 45 minutes; *b*, après 1 h. 10 min.; *c*, après 1 h. 55 min.; *d*, après 2 h. 10 min.; *e*, après 2 h. 25 min. D'après Fry.

hétérogènes des œufs d'amphibiens, comme Herlant l'a figuré incidemment dans des œufs d'oursin activés (Fig. 4, *b*). Dans tous ces cas les chromosomes ont l'aspect habituel, subissent le clivage longitudinal, s'intègrent normalement dans un diaster si un aster accessoire vient s'adjoindre au monocentre, comme je le rappelais plus haut. Il est donc difficile d'admettre qu'ils diffèrent, même physiologiquement, des chromosomes normaux, et la conception de Fry se trouve donc ici en défaut flagrant.

Il y a cependant un moyen d'éliminer cette antinomie: c'est de tenir compte de ce que le noyau ne contient pas seulement des chromosomes, mais aussi un suc hyalin, la caryolymphe, que nous ne parvenons que rarement à colorer mais qui n'en a peut être pas moins une importance physiologique considérable. Mais pour en arriver là, il faut se résoudre à adapter à nos connaissances actuelles, la conception du centrosome que les mémorables travaux de Boveri ont introduite dans la cytologie il y a un quart de siècle.

Cherchons donc à préciser dans quel constituant cellulaire se trouve le principe qui conditionne la dicentrie. Ce sont les phases initiales du développement qui permettent cette analyse par une comparaison attentive des circonstances dans lesquelles l'œuf forme tantôt un monaster, tantôt un amphiaster.

A ce point de vue cytologique, la parthénogénèse est moins instructive que la fécondation, envisagée dans ses modalités naturelles et expérimentales. Dans l'œuf

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[1] Voir à ce sujet Dalcq, 1928, *a*.

parthénogénétique la dicentrie surgit d'emblée; la combinaison signalée plus haut d'un monaster avec un aster accessoire n'est, contrairement à ce qu'avait cru Herlant, qu'un cas exceptionnel (Chambers, 1921; Fry, 1925). Au contraire, dans la fécondation, les images révélatrices abondent, soit qu'il s'agisse d'une entrave mise à la copulation des pronuclei, ou de polyspermie, de fécondation hétérogène, prématurée ou réalisée à l'aide de sperme intoxiqué.

La constatation primordiale est celle-ci: partout où l'on peut comparer, dans un cytoplasme normalement activé, l'évolution du pronucleus femelle et celle du pronucleus mâle, on voit qu'au voisinage du premier se développe une irradiation simple, à laquelle les chromosomes ovulaires s'intègrent bientôt en un monaster, tandis qu'auprès du second surgit une irradiation double, qui donne naissance à une véritable amphiaster.

J'ai exposé dans un ouvrage récent (1928, p. 95) la série des données expérimentales sur lesquelles est basée cette affirmation: les expériences d'isolement des pronuclei chez l'oursin, réalisées par Ziegler (1898), par E. Wilson (1902), par Boveri (1902); les cas d'activation par contact du spermatozoïde, notamment chez *Nereis* (F. R. Lillie, 1923) et chez *Rana fusca* (Dalcq); les résultats obtenus par Belar (1924) dans la "semiparthénogénèse" des Nématodes. J'ai relevé les exceptions apparentes constatées dans l'isolement des pronuclei chez les Gastéropodes (Conklin, 1904) et chez les Tuniciers (Duesberg, 1926), ainsi que dans certaines fécondations hétérogènes (Godlewski, 1911; Bataillon, 1909), auxquelles je reviendrai d'ailleurs brièvement ci-dessous. J'ai montré comment ces cas aberrants s'expliquent sans peine soit par une "activation" de l'ovocentre, soit par la dysharmonie des protoplasmes spermatiques et ovulaires. Pour ne pas allonger inutilement cet article, je me borne ici à cette rapide énumération. Il en ressort donc que l'observateur a pu faire apparaître côte à côte dans un même corps cellulaire monaster et amphiaster. C'est-à-dire que le principe de la dicentrie est passible d'une localisation assez stricte. Mais quiconque a présente à l'esprit la théorie classique du centrome, telle qu'elle ressort des magnifiques observations de Th. Boveri et telle que l'ont souvent exposée ses commentateurs, donnera à cette coïncidence du monaster et de l'amphiaster une explication immédiate: le pronucleus mâle est flanqué de son centrosome, apporté par la pièce intermédiaire du spermatozoïde, et c'est cet organite granulaire qui en raison de ses propriétés intrinsèques détermine la division de l'aster mâle et entraîne ainsi la dicentrie. Mais cette conception, qui a paru longtemps inébranlable, a été remise en question par les observations et les expériences capitales de F. R. Lillie (1903) sur l'œuf de *Nereis*. Non seulement, dans cette forme, la pièce intermédiaire du spermatozoïde reste normalement empêtrée dans le chorion et n'intervient donc pas dans l'éclosion de l'aster spermatique, mais on peut, par une centrifugation adéquate, briser la tête du spermatozoïde au cours de sa pénétration relativement lente dans le cortex ovulaire: un aster se forme quand même centré sur la section basilaire du noyau comme s'il s'y trouvait un centrosome, et il est capable de division. J'ai eu la chance de rencontrer chez la grenouille rousse ce qu'on peut appeler la contre-partie de cette expérience. Ayant fécondé des œufs normaux avec des spermatozoïdes intoxiqués au préalable par la trypaflavine

(méthode de G. Hertwig), j'ai constaté que ce dérivé de l'acridine a pour effet de condenser et de rendre pour ainsi dire insoluble toute la portion axiale du spermatozoïde, c'est-à-dire la chromatine céphalique et le filament central de la pièce intermédiaire du flagelle, avec les centrioles qui y adhèrent. Or, cette altération n'entrave nullement l'édification de l'amphiaster; à la métaphase on trouve à côté des chromosomes ovulaires toute la portion axiale du spermatozoïde, et l'on peut parfois y reconnaître, inchangé, le centriole spermatique. Ainsi d'une part chez *Nereis*, l'absence du centrosome ne prive pas l'œuf de la dicentrie, d'autre part, chez *Rana*, celle-ci surgit sans que le centrosome intervienne.

Et cependant, nombreux sont les biologistes qui hésiteront à opposer ces deux données expérimentales à la théorie si commode, si bien enracinée dans les esprits, du centrosome, organe différencié de la division cellulaire. Ce ne sont, dira-t-on, que deux expériences, en regard d'un grand nombre de faits d'observation! Mais que l'on veuille bien réfléchir à la difficulté extrême de dissocier deux parties aussi intimement unies que le noyau et l'appareil centrosomial du spermatozoïde; l'étonnant est que cette véritable gageure ait pu être accomplie; il a vraiment fallu pour cela que des hasards heureux récompensent l'effort tenace des cytologistes. Si l'on veut bien prendre les faits en sérieuse considération, on dira cependant qu'ils ne sont qu'un processus de suppléance, de régénération, et l'on invoquera volontiers la mystérieuse régulation des blastomères isolés. Déjà Boveri (1900) parlait de régénération à propos des asters accessoires et de la parthénogénèse. Mais est-il licite de faire appel à la régulation, avec la pointe de vitalisme qu'insinue volontiers ce processus, en dehors des cas où toute autre solution est absolument inabordable? Cette notion n'est-elle pas trop troublante pour qu'on ne l'introduise dans la science qu'avec une extrême circonspection? Or, elle ne servirait ici qu'à sauver une théorie certes respectable et qui a été à son heure un instrument de progrès précieux pour la cytologie, alors qu'il suffit de retoucher légèrement cette théorie dans l'esprit physiologique moderne, pour l'adapter à la fois aux données de l'observation et de l'expérience, et lui ôter d'ailleurs ce qu'elle a de trop spécial aux métazoaires.

A quoi se résume, en effet, sa base cytologique? La constatation d'une irradiation astérienne centrée sur la base de la tête spermatique et dans laquelle une différenciation adéquate met en évidence d'abord un, puis deux centrioles; la division progressive de l'irradiation primitive en deux sphérules qui deviennent soit directement, soit après une phase d'effacement, les pôles de la figure mitotique de segmentation. Mais l'expérience de Lillie montre à l'évidence que le centrage de l'aster sur la portion basilaire du noyau mâle dépend en réalité de la polarité de ce noyau, et que sa relation avec la situation de la pièce intermédiaire n'a donc que la valeur d'une coïncidence. Pour être tout à fait impartial et réserver quand même le rôle éventuel, encore que problématique, du ou des granules centriolaires[1] du spermatozoïde, il suffit de dire que le principe de l'irradiation et de la dicentrie réside dans le noyau mâle, mais que le grain préformé peut amorcer et localiser l'irradiation, dont la cause est cependant en dehors de lui. Ainsi, un germe cristallin jeté dans une eau-mère

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[1] La valeur réelle de ceux-ci est plus probablement d'être des organites de la différenciation du flagelle, conformément à la relation sur laquelle Henneguy a beaucoup insisté.

amorce une solidification dont les conditions étaient préalablement réalisées dans le milieu. Il n'en reste pas moins, objectera-t-on encore, que la scission de l'aster primitif est parfois précédée de l'apparition d'une centriole double qui semble bien témoigner de ce que le *primum movens* de la division gît dans l'intimité du centrosome. C'est évidemment une possibilité, mais avant de se rallier à cette interprétation granulaire qui limite inexorablement l'exploration d'un phénomène capital, il faut examiner si elle est la seule satisfaisante. Or, il n'est pas douteux que l'image du double centriole est un aspect limite, où la coloration régressive a sa large part. Si l'on considère les meilleures figures de "division" du centriole, et spécialement celles de la grande monographie de Boveri (1900), on y verra que bien souvent (chez *Diaulula*, *Echinus*, *Ascaris*) les centrioles sont les extrémités colorables d'une tigelle plus ou moins renflée de substance hyaline, partie centrale du "Netrum" ou Centralspindel de Boveri. Il est possible qu'avec une décoloration poussée cette portion interposée échappe à l'examen. Son existence dans les cas les plus clairs porte à croire que ce qui nous en impose généralement comme une division soudaine c'est en réalité l'élaboration au centre de l'astrosphère d'un corpuscule allongé dont l'axe principal crée la dicentrie. Et la remarque que nous formulons ici à propos de la dicentrie initiale de l'œuf s'applique aussi à toute division du centrosome. Chaque fois que l'on suit celle-ci de près, soit à l'ana-télophase des cinèses de segmentation à succession rapide, soit dans certaines anomalies de la maturation[1], on voit qu'elle procède d'une élongation interne. Mais ce phénomène, qui évoque la formation d'une sorte de cristal liquide ellipsoïdal, ne saurait être envisagé comme strictement autonome. Il est le produit d'une interaction entre les substances propres à l'astrosphère et d'autres qui affluent vers elle. C'est là l'avantage pratique du tempérament apporté au point de vue strictement morphologique. Nous sommes maintenant en droit de nous demander pour l'aster spermatique, d'où lui vient la cause de la dicentrie, et grâce aux expériences d'isolement des pronuclei, d'en localiser la source dans le noyau spermatique ou le pronucleus qui en dérive. C'est, si l'on veut, remettre en honneur la notion un peu désuète du centronucleus que Boveri admettait déjà pour l'œuf parthénogénétique et pour les Protistes et les cellules végétales dépourvus de centrosome différencié ; mais nous y introduisons la distinction essentielle entre la production d'un gel astérien centré et le principe de la dicentrie : la propriété gélifiante est commune, à un stade donné du cycle cellulaire, aux pronuclei mâle et femelle ; le principe de la dicentrie est, dans l'œuf fécondé, localisé au pronucleus mâle ; il sort ses effets par l'interaction entre celui-ci et le cytoplasme *activé*[2] ; et nous allons même voir que cette activation est indispensable pour qu'il se manifeste.

Car sur ce terrain, l'analyse peut être prolongée bien davantage. On peut d'abord distinguer des indications de ce que le principe de la dicentrie n'entre en jeu qu'à la condition qu'il existe entre le pronucleus mâle et le protoplasme ovulaire une certaine harmonie. S'il est vrai que dans beaucoup de fécondations hétérogènes

[1] Voir mon mémoire de 1924 sur la dépolarisation de l'œuf d'*Asterias glacialis*.

[2] J'ai montré ailleurs la nécessité de distinguer l'activation, simple éveil du métabolisme ovulaire, de la parthénogénèse, qui implique précisément l'acquisition de la dicentrie par le noyau ovulaire et la perpétuation de cette propriété nouvelle (1928, *a*).

la dicentrie s'installe sans accroc, on connaît par exemple l'imprégnation de l'œuf d'oursin par le sperme de chætoptère (Godlewski, 1911), qui ne confère à l'œuf que l'activation simple, avec cycle monastérien, sauf si l'on fait intervenir un traitement adjuvant (NaCl hypertonique). Ainsi, l'entrée en jeu du principe de la dicentrie, sans être strictement spécifique, est cependant limitée par la dissemblance des protoplasmes.

D'autre part, diverses observations tendent à montrer que le principe de la dicentrie ne devient efficace qu'à la condition que le cytoplasme ovulaire soit réellement activé. Grâce aux belles observations de Brachet (1922) on connaît depuis quelques années les particularités de la "fécondation prématurée." Celle-ci consiste dans l'invasion de spermatozoïdes dans les œufs bloqués en voie de maturation et qui le restent (Fig. 7). Elle a surtout frappé par le phénomène de "mise à
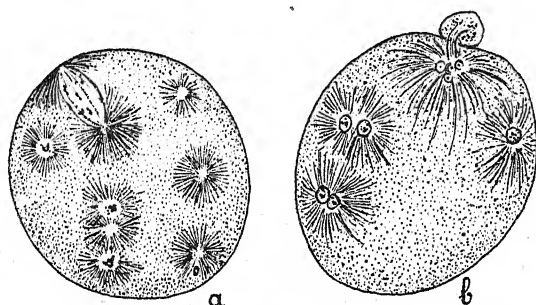
Fig. 7. *a*, Œuf d'oursin bloqué en métaphase II; nombreuses têtes spermatiques résolues en chromosomes, avec aster non dédoublé; *b*, phase de reconstitution des pronuclei. Absence de toute dicentrie. D'après Brachet.

l'unisson" des noyaux spermatiques avec la figure de maturation, et j'en ai montré ailleurs l'intérêt au point de vue de la "dépolarisation physiologique" de l'œuf en maturation (1928, *a*). Mais c'est un autre aspect de ce phénomène si instructif qui doit nous retenir ici. Quatre formes en sont actuellement connues: outre l'observation originale faite chez l'oursin, Bataillon (1927) l'a rencontré dans des croissements de Batraciens et dans des œufs de ver à soie (Bataillon et Tchou-su, 1928), tandis que j'en réussissais l'imitation expérimentale dans les œufs d'astérie bloqués artificiellement (1923, 1924). Or, j'ai noté au cours de mes propres observations l'absence de membrane de fécondation. D'autre part, il est constant que la cinèse de maturation reste figée et de plus, tant chez les Batraciens que chez les Échinodermes, le réglage de la monospermie fait défaut, l'invasion spermatique se prolonge pendant un temps considérable. Trois des manifestations les plus caractéristiques de l'activation, le soulèvement de la membrane, l'achèvement de la maturation, le réglage de la monospermie, font donc défaut. On peut en conclure avec certitude *que ces œufs ont été fécondés sans être activés.* Cette remarquable dissociation entre la captation du gamète mâle et l'activation nous montre en même temps une absence ou une atypie marquée de la dicentrie. Chez les Échinodermes, tous les

asters mâles des œufs bloqués restent monocentriques[1] (Fig. 7). Chez les Batraciens, c'est, ou bien la monocentrie, ou bien la figure anastrale en tonnelet trapu, analogue à certaines cinèses de maturation, et qu'on a parfois rencontrée aussi dans les monasters femelles (p. 189). Chez le ver à soie, l'anomalie est différente, la dicentrie apparaît, mais le fuseau s'étire démesurément et il s'ensuit une ébauche de plasmodiérèse accompagnée d'irradiation plasmatique. En somme, le principe de la dicentrie ne sort donc ses effets typiques qu'en interaction avec un cytoplasme activé; il est d'ailleurs possible aussi, l'activation étant probablement corticale, que le spermatozoïde soit affecté en quelque manière pendant qu'il traverse la couche corticale en plein remaniement et qu'il subisse réellement alors cette "activation mâle" que F. R. Lillie a suggérée.

Mais il ne s'agit pas ici de l'essence de l'activation. Nous ne visons qu'à rencontrer les modifications expérimentales de la cinèse en les groupant de manière à éclairer l'énigme de la dicentrie. S'il est admis que dans l'œuf normalement activé son principe réside dans le pronucleus mâle et que le grain centrosomial éventuel n'intervient que dans sa localisation, nous pouvons essayer de franchir la dernière étape de cette analyse et examiner si ce principe est la propriété globale du pronucleus mâle, ou si on peut l'attribuer à un constituant défini de ce dernier. Au moment d'une cinèse, il est permis d'y distinguer d'une part les chromosomes en lesquels se concentre l'acide thymonucléique de la cellule et, d'autre part, un liquide abondant, la caryolymphe avec ses nucléoles éventuels. Ce suc nucléaire disparaît à nos yeux dès que la membrane est résorbée et nous ne savons s'il reste sur place ou s'il diffuse dans le cytoplasme. Remarquons cependant que certaines observations de cytologie végétale (Robyns, 1924–26) révèlent qu'il constitue en grande partie le fuseau. Trois constatations conduisent à admettre que le principe de la dicentrie n'est pas contenu dans les chromosomes: 1º. Dans les fécondations hétérogènes, comme dans les cas de semi-parthénogénèse, les chromosomes mâles sont éliminés à un stade plus ou moins précoce, sans préjudice de l'établissement d'un amphiaster normal. 2º. Dans l'irradiation du spermatozoïde (O., G., et P. Hertwig) ou dans son intoxication par la trypaflavine, un pronucleus mâle de volume normal se forme, bien que la tête spermatique reste compacte (Fig. 8) et soit éliminée dès la première cinèse; un amphiaster s'édifie cependant, même s'il n'y a pas eu copulation avec le pronucleus femelle, comme c'est le cas pour les spermatozoïdes supplémentaires dans les œufs polyspermiques de *Rana fusca*. 3º. La polyspermie expérimentale de la grenouille rousse pratiquée avec les spermatozoïdes intoxiqués à point par la trypaflavine, permet de dissocier avec la plus grande netteté le rôle des chromosomes et celui du suc nucléaire. On sait en effet par les travaux de Brachet (1911) et Herlant (1912) que chez cette espèce une polyspermie légère est suivie du partage de l'œuf en une série d'énergides dans lesquelles s'édifient des cinèses indépendantes. L'une d'entre elles est diploïdique, d'origine amphimixique, les autres sont haploïdiques, purement spermatiques; il s'agit donc d'une nouvelle modalité expérimentale de la cinèse, permettant de comparer dans un même corps

[1] Souvent avec le curieux aspect de figure en éventail, tous les chromosomes étant groupés d'un seul côté de l'aster. Bataillon signale la même particularité chez les Batraciens.

cellulaire le diaster normal à un diaster haploïdique mâle ; Herlant (1913) a attiré l'attention sur la brièveté relative de la seconde figure. Or, si l'intoxication préalable du spermatozoïde a été suffisante, sa chromatine va rester en masse compacte,
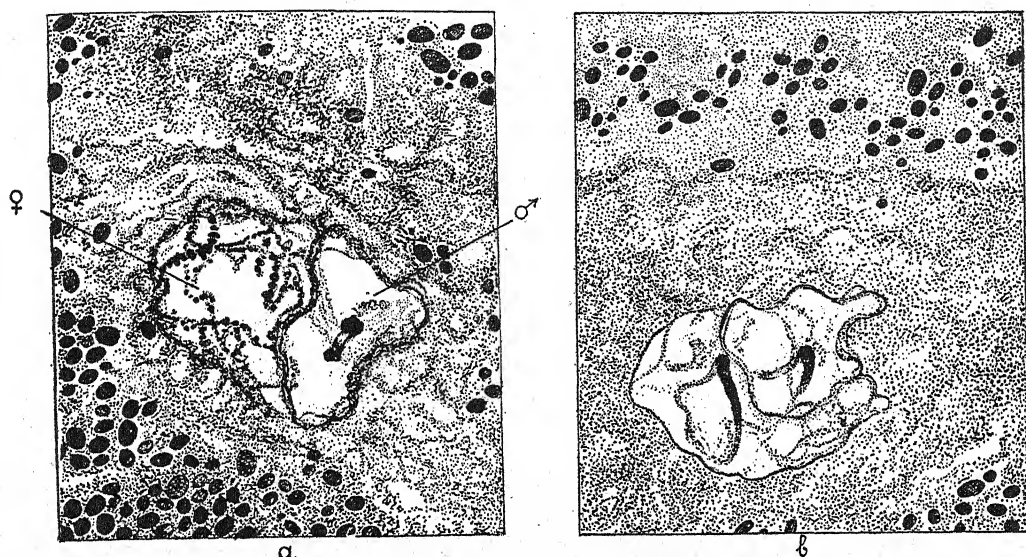
Fig. 8. Œuf polyspermique de grenouille, fécondé par des spermatozoïdes trypaflavinés, fixé 1 h. ½ après la fécondation. *a*, pronucleus femelle et pronucleus mâle accolés, ce dernier avec la masse compacte de chromatine spermatique ; *b*, deux autres pronuclei mâles ayant copulé avant de former un aster, suivant la loi de Brachet ; dans chacun d'eux la tête spermatique compacte.

comme elle l'était dans la tête spermatique, elle occupera seule l'équateur des cinèses d'origine purement spermatique et, lors de l'anaphase, passera tout entière à l'un des pôles, à moins qu'elle ne soit d'emblée éliminée dans le cytoplasme. Dans l'un et l'autre cas, il en résultera l'apparition de un ou plusieurs pôles dépourvus de tout
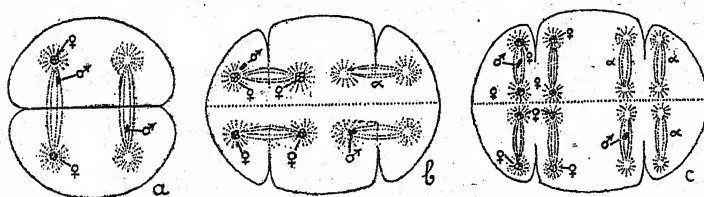
Fig. 9. Schéma de l'évolution d'un œuf de grenouille fécondé par deux spermatozoïdes trypaflavinés. *a, b, c*, premier, deuxième et troisième cycles mitotiques ; ♀, noyaux haploïdiques issus du pronucleus femelle ; ♂, masse chromatique des spermatozoïdes ; *a*, cinèses achromosomiales.

chromosome comme le montre le schéma de la Fig. 9, dans l'éventualité la plus simple d'un œuf dispermique.

Le but de l'expérience était de voir si ces astrosphères dépourvues de chromosomes continueraient à se diviser. Le contrôle cytologique—dont on connaît les difficultés—a permis d'établir dans onze œufs qu'il en est effectivement ainsi (1927).

J'en donne comme exemple la Fig. 10, reconstitution fidèle de la répartition des cinèses dans un œuf dispermique parvenu à la fin du troisième cycle mitotique. La comparaison avec la Fig. 9 montre que le résultat correspond étroitement aux prévisions cytologiques. Je fais abstraction ici de la relation entre les cinèses achromosomiales et la plasmodiérèse, ainsi que de l'épuisement assez rapide de leur activité. Le point essentiel est que ces cinèses existent, au moins pendant deux cycles complets, qu'elles possèdent un fuseau et des irradiations polaires. Il en résulte que le principe actif de la dicentrie ne se trouve pas dans les chromosomes[1]. Il ne peut donc résider que dans le suc nucléaire ou dans des centrosphères elles-mêmes. Ici surgit naturellement une dernière difficulté d'interprétation. On pourrait dire que l'expérience ne montre rien de plus que l'autonomie fonctionnelle du
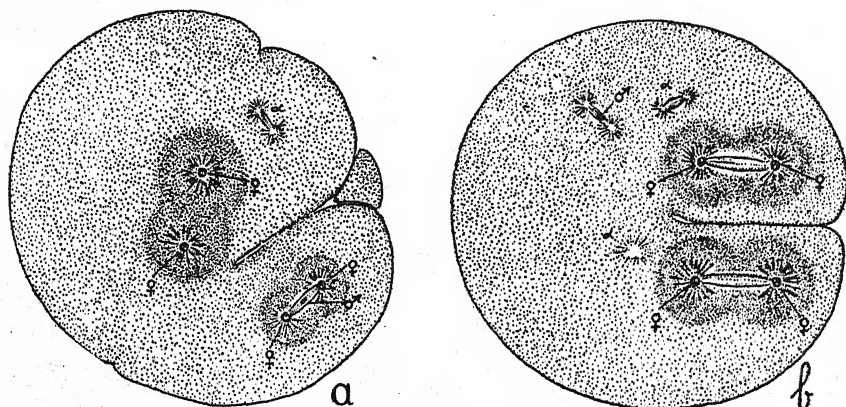
Fig. 10. Reconstitution en deux parties d'un œuf de grenouille fécondé par deux spermatozoïdes trypaflavinés et montrant la répartition des cinèses avec et sans chromosomes ainsi que les deux têtes spermatiques. Mêmes indications que dans la Fig. 9.

centrosome. Mais je crois avoir montré plus haut combien il est plus rationnel et plus fécond de considérer que le centrosome *subit* la division plutôt qu'il ne la suscite. Et d'autre part l'attribution du principe actif au suc nucléaire permet seule de concilier toutes les données relatives à cette question. Dans l'hypothèse de l'autonomie du centrosome, comment comprendre que les asters accessoires dépourvus de chromosomes ne se divisent pas, ne forment pas de fuseau, alors que cela se produit, en l'absence également certaine de chromosomes et dans les cinèses achromosomiales de la grenouille et dans les œufs énucléés de McClendon, sans parler des résultats non négligeables de Boveri et de Ziegler? L'explication de ces différences c'est que là où l'on voit des chromosomes il y a eu, aux stades précédents, un noyau qui s'est rompu et a imbibé le cytoplasme de son suc. Entre les expériences comparables de McClendon[2] et de Fry, la différence majeure est

[1] Certains faits indiquent cependant que les chromosomes ne sont pas étrangers à son maintien ou à sa perpétuation.

[2] McClendon a clairement discerné l'importance du suc nucléaire, mais il a cru à un pouvoir spécial—d'ailleurs possible—du suc de la vésicule germinative. "Apparently," écrit-il, "the formation of the cytasters necessary for cleavage requires the presence of the nuclear sap, which is formed in the presence of chromatin. It is possible that the final condition of rest in the operated eggs is due to the disappearance of some substance from the nuclear sap" (p. 667).

que l'un opère sur des œufs en phase de cinèse maturative, tandis que l'autre agit sur des ootides à pronucleus intact et les sectionne en respectant ce dernier. Enfin,
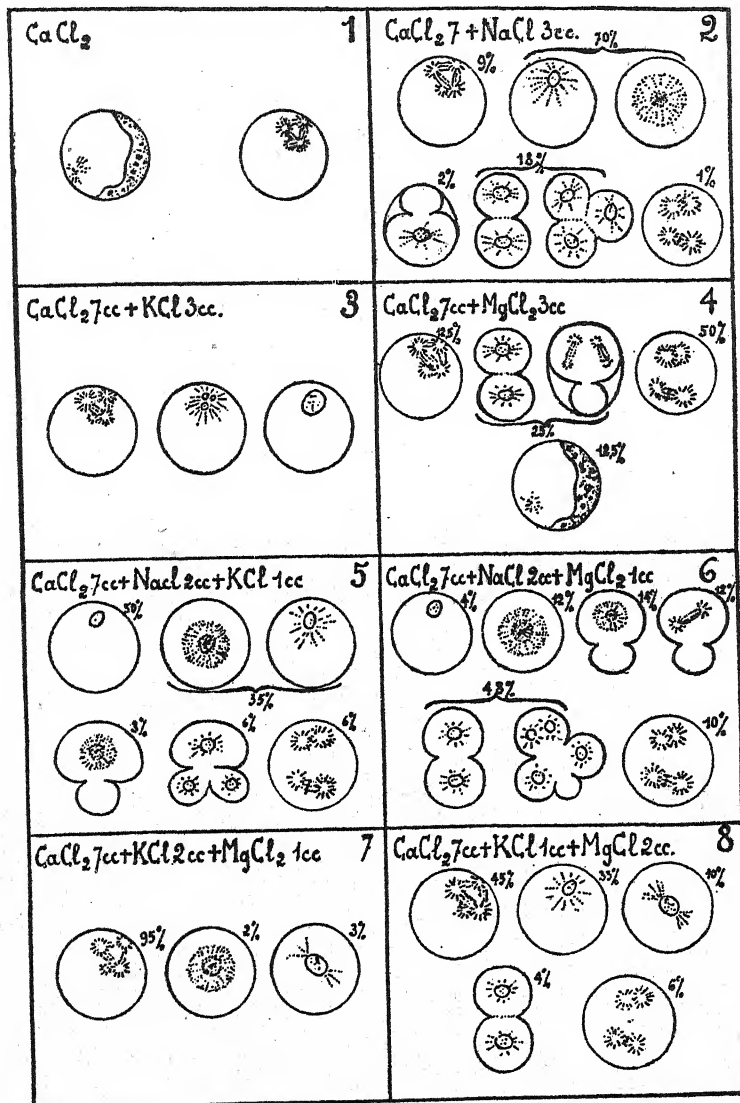
Fig. 11. Représentation schématique d'une série de préparations obtenues par la culture des oocytes vierges d'*Asterias glacialis* dans huit solutions dont la composition est indiquée dans chaque case. Fixation au bout de 6 heures.

ce qui caractérise l'œuf vierge purement activé, ce qui l'engage dans "l'impasse des cycles monastériens[1]," c'est la carence de son suc nucléaire quant au principe actif de la dicentrie. Et c'est en ce sens alors que la parthénogénèse reprend tout son

[1] Bataillon.

intérêt, parce qu'elle peut nous faire connaître une relation entre des conditions expérimentales et l'avènement de la dicentrie. Dès à présent, il existe un document exploitable en ce sens, c'est l'expérience de comparaison de l'efficacité parthénogénétique des quatre cations principaux de l'eau de mer, telle que je l'ai réalisée en 1923 sur les œufs d'*Asterias glacialis*; il en ressort que le Mg aurait, tout au moins dans ce cas particulier, une importance spéciale au point de vue de l'acquisition de la dicentrie (Fig. 11). (Voir à ce sujet, p. 202.)

En résumé, au point où nous en sommes arrivés, l'analyse expérimentale de la cinèse nous impose la distinction entre la gélification radiée, réaction assez banale du cytoplasme, et l'élongation interne de ce système qui conduit à la bipartition de l'astrosphère. Il est remarquable que cette élongation a ses formes atypiques, telle que cette mitose bipolaire anastrale qui surgit assez souvent à la phase du monaster. On est ainsi conduit à soupçonner un phénomène d'orientation moléculaire; le suc nucléaire, prêt à la cinèse, pourrait contenir ou donner par réaction avec le cytoplasme une substance complexe, se concentrant dans la sphère de l'irradiation; selon sa structure moléculaire, elle resterait à l'état de globule comme dans le monaster ou formerait un ellipsoïde plus ou moins allongé[1]; ainsi de minimes différences chimiques, portant sur quelque chaînon de cette substance complexe, entraîneraient d'énormes conséquences dynamiques[2]. Et si l'on veut pousser la question jusqu'à ces dernières déductions théoriques, on pourrait voir dans la division anaphasique des centrosphères un indice de ce que le suc nucléaire, qui tend à prendre la dignité d'un constituant essentiel de la cellule, reflue à ce moment vers les pôles pour participer à la reconstitution des caryomères et ainsi des noyaux-filles. Ce que l'on sait aujourd'hui de la formation du pronucleus mâle (cf. Fig. 8) atteste que les phénomènes de ce genre ne se réduisent pas, comme on le pensait jusqu'ici, à une banale imbibition de la chromatine.

Cet exposé montre bien quelle est l'attitude qui s'impose, à mon sens, à l'égard de la théorie de Boveri. Il ne saurait s'agir de nier l'importance du centrosome dans l'édifice cellulaire; n'y aurait-il en sa faveur que les phénomènes de centrotaxie de la prématuration que cette importance resterait de premier ordre. Mais il est permis de chercher à assouplir une conception trop rigide, afin de rouvrir à l'analyse expérimentale une voie qui paraît féconde. En ce sens, on embrasse aisément les faits en considérant qu'au point de vue de l'édification de la cinèse le centrosome permanent éventuel ne fait que localiser dans le cytoplasme une modification dont la cause est en réalité dans le suc nucléaire.

Le véritable organe de la cinèse est bien moins le centrosome que le gel fusorial ou ses équivalents. Le centrosome fait défaut dans bien des figures, et Boveri ne le considérait d'ailleurs que comme un perfectionnement secondaire de la mitose chez les Métazoaires. Cette conception est difficilement soutenable, car de magnifiques centrosomes, avec irradiation, s'observent dans des divisions de Flagellés, en lesquels on s'accorde à voir les plus primitifs des Protozoaires, tandis qu'on

[1] Il est connu que les cristaux liquides peuvent prendre des aspects de fuseau. Cf. Mauguin, 1921.

[2] A l'appui de cette suggestion, rappelons que le simple secouage fait se substituer temporairement le monaster à la dicentrie dans les œufs d'oursin (Th. Painter, 1916).

rencontre çà et là des cinèses anastrales chez les Métazoaires. En réalité, il semble bien que ce soit une circonstance d'ordre secondaire qui fait que des travées radiaires se dessinent ou non autour des pôles mitotiques, de même qu'il paraît assez indifférent que la membrane nucléaire s'efface ou persiste. Le fait capital, constant, qui avec l'apparition des chromosomes caractérise la division des Eucaryotes (Chatton), c'est l'existence d'une gélification allongée, formée en majeure partie de substance nucléaire, qui constitue le fuseau avec ses multiples variantes, et crée le grand axe de polarité cinétique[1]. Pour aller au fond de ce problème, il faudrait envisager les rapports du centrosome des Flagellés avec le fouet au cours de la cinèse, avec les remarquables dissociations que présentent certaines formes entre la caryocinèse et la division du complexe fouet-centrosome. On pressent là des relations philogénétiques du plus haut intérêt, mais dont malheureusement la filiation exacte est difficile à reconstituer.

Si nous récapitulons maintenant, en dehors de toute préoccupation théorique, les modifications expérimentales de la cinèse que nous avons rencontrées jusqu'ici, nous pourrons les énoncer ainsi:

Asters accessoires.

Cycle monastérien.

Combinaison d'un cytaster et d'un monaster en un amphiaster.

Fuseau anastral.

Coexistence d'un monaster et d'un amphiaster.

Coexistence de deux ou plusieurs amphiasters amphimixique et haploïdique.

Cinèses achromosomiales.

Ce n'est pas tout. Mentionnons d'abord les innombrables cas de polycentrie expérimentale, qu'ils proviennent de la polyspermie ou d'une intoxication de la cellule (O. et R. Herwig). Signalons ensuite la possibilité d'un remaniement considérable de l'édifice métaphasique. C'est ainsi que si l'on entrave l'expulsion du premier globule polaire dans un œuf d'astérie on peut parfois assister simplement à l'évolution interne de la première cinèse, mais plus souvent on peut constater un étirement transversal suivi de dédoublement d'un ou des deux pôles et la transformation directe en métaphase polycentrique. D'après l'aspect des chromosomes, celle-ci est d'abord de premier ordre, mais ultérieurement le dédoublement de ces éléments peut se produire sur place et ceux-ci perdent l'aspect hétérotypique. Ajoutons enfin que l'on observe souvent dans les cinèses bloquées une augmentation de volume des centrosomes, dont Bataillon (1910) a figuré de beaux exemples dans son étude des œufs asphyxiques d'*Ascaris*, et que l'on peut d'autre part provoquer l'effacement des irradiations astériennes soit par les narcotiques tels que l'éther (E. B. Wilson, 1901; Gray, 1925) ou par la privation d'oxygène (Mathews, 1907; E. B. Harvey, 1927); ces transformations sont réversibles dans certaines limites.

[1] Le processus d'allongement et d'étirement du "caryosome" ressort remarquablement des images que A. Kuhn (1920) a données de la division de *Vahlkampfia bistadialis*, soumise à une légère compression.

Enfin, de nombreux essais pratiqués sur les cellules végétales et animales ont provoqué dans la disposition généralement si équilibrée des chromosomes des troubles qui établissent une série de transitions entre l'amitose et mitose. Les botanistes (Nemec, Wasieliewski, etc.) ont surtout employé les narcotiques et récemment Wettstein (1923) a fait une ingénieuse application de cette technique à la production d'individus polyploïdes chez les mousses. Dans ces dernières années Alberti et Politzer (1924), puis Politzer (1924), ont raffraîchi l'intérêt cytologique de cette question par d'intéressantes études sur l'épithélium cornéen de larves d'Urodèles. Ces auteurs ont fait apparaître dans ce tissu toute la gamme des pseudo-amitoses par les Rayons X, la lumière ultra-violette, le rouge neutre, etc. Mais jamais jusqu'ici on n'a pu obtenir de véritables amitoses; toujours la prophase reste normale avec l'individualisation caractéristique des chromosomes; ce ne sont donc que des pseudo-amitoses.

CARYOCINÈSE ET PLASMODIÉRÈSE.

On sait que la division du corps cellulaire s'effectue suivant deux modes distincts Dans les cellules à paroi plus ou moins rigide, très répandues dans le règne végétal, la cloison nouvelle paraît se former grâce à l'activité d'un organite spécial, le phragmoplaste, qui apparaît secondairement sur le fuseau. Dans les cellules à paroi molle, c'est un sillon qui se creuse dans la partie médiane, suivant un plan perpendiculaire au fuseau, et la division rappelle celle d'une gouttelette suspendue.

Le premier mode n'a guère encore été touché par l'expérimentateur. Signalons cependant que d'intéressantes observations ont été faites par Conard (1922–26) au cours de la cicatrisation des tissus de *Haga Carnosa* et de *Tradescantia virginica*. Et rappelons que c'est sur ce dernier matériel que Demoor (1894) a réussi la première dissociation entre la caryo- et la plasmodiérèse.

Le second mode est celui qui se rencontre dans les cellules libres et une fois de plus, les œufs des diverses espèces ont fourni sur ces points d'abondants renseignements.

On est parvenu à affecter leur plasmodiérèse au moins de trois manières; en la supprimant, en la modifiant, et en faisant apparaître des formes atypiques.

La suppression de la plasmodiérèse s'obtient par des moyens multiples portant sur les propriétés les plus diverses du milieu: pression osmotique, pH, température, constitution saline, teneur en CO_2, l'absence de O_2, présence de narcotique, etc. La liste des circonstances où on l'a observée serait fort longue et n'aurait qu'un intérêt minime. Généralement, elles ont pour simple résultat de maintenir la cellule dans sa forme primitive, tandis que le noyau poursuit au moins pendant quelque temps son rythme cinétique. Mais si l'on modifie plus profondément le milieu en lui soustrayant la plus grande partie de ses électrolytes, par exemple en immergeant les œufs dans une solution de saccharose ou de glucose isotonique à l'eau de mer, on obtient des formes différentes, amiboïdes (R. S. Lillie, 1902).

Il n'y a guère à attendre de modification de la plasmodiérèse dans la segmentation, si ce n'est qu'on peut parfois différer la séparation des territoires cellulaires, comme cela s'observe dans l'action de l'éther sur les œufs d'oursin (E. B. Wilson). Mais dans la maturation, on peut chercher à faire varier le volume des globules polaires

ou à transformer le processus en une division de segmentation. Chez l'étoile de mer, j'ai obtenu la formation de globules géants (1º) par l'eau de mer diluée à demeure; (2º) par des mélanges de chlorures où $MgCl_2$ et surtout $CaCl_2$ prédominaient; (3º) par un passage dans une solution hypertonique, suivi du retour au milieu normal. Des résultats analogues avaient d'ailleurs été signalés par Miss King (1906), par compression des œufs, et incidemment par divers chercheurs s'occupant de parthénogénèse. J'ai vu aussi la seconde figure de maturation prendre, spécialement en présence du $CaCl_2$, une orientation tangentielle et imiter d'assez près un clivage total et égal (Fig. 12). Heilbrunn (1925) chez *Crepidula*, et Fauré-Frémiet chez *Sabellaria*, affirment avoir vu dans la parthénogénèse thermique la première figure de maturation inaugurer le clivage de l'œuf en se substituant à la cinèse de
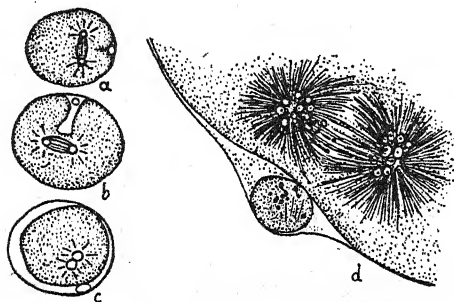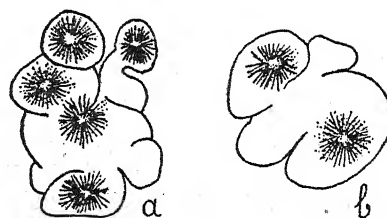
Fig. 12.   Fig. 13.

Fig. 12. Œufs d'astérie cultivé dans $CaCl_2$. *a, b, c*, croquis successif d'un œuf *in vivo* montrant l'orientation tangentielle de la seconde figure de maturation et la tentative de division; *d*, coupe d'un œuf analogue, avec télophase de la seconde cinèse en orientation tangentielle.

Fig. 13. Coupes de fragments anucléés d'œuf de scutelle traitées par la méthode de Loeb. *a*, après 25 min.; *b*, après 1 h. 5 min.; tentatives irrégulières de clivage. D'après Fry.

segmentation. Les formes anormales de la division du corps cellulaire sont spécialement intéressantes au point de vue des relations entre la caryocinèse et la plasmodiérèse. En effet, la disposition fascinante de l'édifice mitotique et la chronologie des évènements de la division indirecte imposent pour ainsi dire l'idée que la plasmodiérèse est la conséquence de la caryocinèse. Et si l'on considère alors les images observées par McClendon (Fig. 5) on est tenté d'admettre un rôle prédominant de la figure achromatique. Et ici encore, la présence d'un fuseau, probablement liée à celle du suc nucléaire, semble avoir son importance car les fragments énucléés de Fry (p. 188) ne se divisent que dans 11 per cent. des cas, et encore de façon tout à fait atypique et sans aucune tendance à la continuation du clivage (Fig. 13). Il faudrait cependant se garder d'en déduire que le sillon de division ne se peut creuser que perpendiculairement à un fuseau établi. Il existe divers exemples où le plan de clivage s'est formé entre deux édifices mitotiques situés dans le même corps cellulaire. La relation entre la figure achromatique et la plasmodiérèse n'est donc pas d'ordre morphologique; les rétractions des filaments gélifiés du cytoplasme n'y entrent que pour une part minime, et il est difficile actuellement de généraliser les relations physiques si séduisantes qui semblent se dégager des faits

de perméabilité, de consistance du cytoplasme, de courants internes et de tension superficielle[1].

En réalité, la plasmodiérèse jouit à l'égard de la caryocinèse d'une autonomie bien plus grande qu'on ne pouvait le soupçonner, ainsi qu'il ressort de la considération des faits suivants: Jollos et Peterfi (1923) ont réussi à enlever à l'aide d'une micropipette le noyau de l'œuf fécondé de l'Axolotl et ont vu la segmentation se continuer d'une manière assez régulière jusqu'au stade morula ou jeune blastula. L'étude cytologique de cet intéressant phénomène est malheureusement peu complète. Au cours de ses recherches fondamentales sur les localisations germinales de l'œuf de Dentale, E. B. Wilson (1904) a procédé à l'excision du lobe polaire formé au cours de la première division de cet œuf et a constaté qu'il continuait à se comporter comme s'il était en place; l'éminent biologiste a vu ce fragment purement
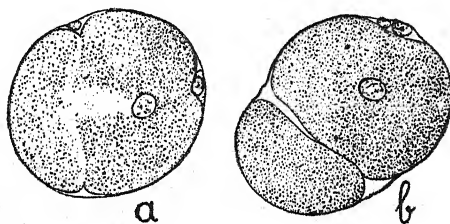
Fig. 14. Œufs d'astérie cultivés dans un mélange de
chlorures et montrant l'autotomie végétative.

plasmatique, sans irradiation ni noyau, reformer une sphérule indivise, puis montrer un nouvel étranglement lobaire et finir par se scinder en deux fragments. Et si l'on voit ainsi la plasmodiérèse—ou du moins un processus qui lui paraît bien équivalent—se faire en l'absence du noyau, on peut aussi l'observer dans un corps cellulaire entier dont le noyau reste parfaitement quiescent, dont le cytoplasme ne décèle aucune irradiation; c'est ce qui se passe dans l'autotomie végétative que j'ai rencontrée au cours de mes recherches sur la parthénogénèse de l'œuf d'astérie dans les milieux calciques (Fig. 14).

Ces recherches ont montré aussi, et c'est un point qui pourrait être important pour l'analyse ultérieure de ces phénomènes, que l'activité de déformation du cytoplasme est dans une large mesure contrôlée par la composition du milieu. Dans un mélange des quatre chlorures avec prédominance de $CaCl_2$, une même ponte peut montrer selon la sensibilité individuelle de ses œufs, le repos complet à l'état sphérique, ou les lobulations désordonnées qui accompagnent le cycle monastérien, ou l'autotomie suivie de segmentation partielle, ou la vraie segmentation ou encore la caryocinèse sans clivage. Or, si l'on répartit les œufs vierges d'une même ponte dans une série de mélanges contenant une même quantité de $CaCl_2$ et de quantités variables de NaCl, KCl et $MgCl_2$ et qu'on suive de près toute l'évolution de ces cultures, on obtient le résultat que résume la Fig. 11. Il est surtout à noter que

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[1] (Note ajoutée lors de la correction.) Des expériences encore inédites, réalisées ce printemps sur l'œuf de grenouille, m'ont montré que les chromosomes jouent un rôle important et tout à fait inattendu dans la plasmodiérèse.

l'activité corticale est plus grande avec Na qu'avec Mg et que le K exerce à ce point de vue une influence frénatrice caractéristique.

CONCLUSION.

Il n'y a plus guère de processus inhérent à la division cellulaire que nous ne soyons en mesure de toucher, de modifier ou même de susciter à notre gré. Seulement, nous ne pouvons pas toujours atteindre ce résultat chez une seule et même forme. Nous sommes encore forcés, et sans doute en sera-t-il encore longtemps ainsi, de nous adresser aux types cellulaires qui se prêtent le mieux, de par certaines dispositions particulières, à nos diverses interventions. Mais cette réserve, qui s'atténuera certainement encore avec les progrès de l'expérimentation, n'a pas une importance très grande. A de très rares exceptions près, il n'y a en effet aucune notion dégagée de ces multiples investigations qui détonne dans l'ensemble. Tout porte à croire que sous des variantes de détail un processus aussi banal, aussi fondamental que la division cellulaire, obéit à des lois absolument générales. Ces lois sont-elles connues? Il serait exagéré de le prétendre! Certes, les aspects cytologiques de la division nous sont familiers dans la plupart des cas. Mais cette exploration préalable permet tout juste de dégager, comme j'ai tenté de le faire plus haut, ce que les divers types de cinèse ont de commun et sans doute de fondamental. Nous savons aussi que l'apparition de la cytodiérèse est liée à certaines conditions extérieures: pH, constitution du milieu, échanges respiratoires, etc....qui réalisent un simple optimum de la vie cellulaire. Et c'est précisément à ces notions que devraient se relier les lois physiologiques de la division. Peut-être n'est-il pas inutile, à titre d'orientation, de supputer ce qu'elles pourraient être. Si diverses que soient les circonstances (croissance, fécondation, induction, etc.) qui amènent une cellule à se diviser, elles doivent susciter dans le protoplasme quelque chose de commun qui entraîne la division. La première loi devrait donc définir la nature des conditions qui, une fois réunies dans une cellule quelconque, déclenchent sa division. Il doit d'autre part exister un facteur ou un groupe de facteurs, qui font que la division ainsi déterminée sera mitotique ou amitotique. Cet énoncé pourrait faire l'objet d'une seconde loi. En nous plaçant dans l'hypothèse de la mitose, mode fondamental de la cytodiérèse de tous les Eucaryotes (Chatton), il faudrait alors pouvoir préciser pourquoi la membrane nucléaire disparaît ou persiste selon les formes; pourquoi les chromosomes s'individualisent à la prophase, pourquoi les pôles de la figure mitotique sont ou non entourés d'une gélification radiée; pourquoi la cellule en intercinèse possède un centrosome ou n'en montre point. A côté de ces diverses lois, d'autres devraient nous apprendre d'où dépend la monocentrie, la dicentrie ou la polycentrie, ou plus exactement—puisque les centres ne sont qu'un aspect secondaire—à quelles conditions obéit la polarisation de l'édifice mitotique. Il resterait à envisager toute la question de la statique et des transformations des chromosomes, de la relation entre la caryo- et la plasmodiérèse, de la séparation des cellules-filles par déformation corticale ou par élaboration d'une cloison, etc. Mais je ne veux ici qu'esquisser un canevas qui suffit à montrer tout ce que ce domaine attend encore des chercheurs. En réalité, le but est peut-être moins lointain qu'on

ne l'imagine. Il n'y a presque aucun des problèmes que devraient résoudre les lois à venir qui n'ait été comme on vient de le dire dès à présent abordé et où l'expérience n'ait prise de quelque manière. Le terrain d'attaque est donc préparé, il suffira d'y consacrer l'effort nécessaire. Et ce n'est pas aux cytologistes qui liront cet article qu'il faut en justifier l'utilité théorique et pratique.

Qu'il me soit permis, en terminant, d'énoncer quelques remarques qui pourraient, me semble-t-il, trouver leur application dans les investigations futures. J'ai posé, en commençant cet article, la distinction entre les processus intrinsèques de la division et les phénomènes de croissance. Cette distinction était nécessaire pour limiter mon sujet et éviter un double emploi avec d'autres mises au point, mais il est clair qu'elle n'a pas d'autre valeur. Selon l'aphorisme de Spencer, il existe au contraire une relation foncière entre l'assimilation et la division. Et dans les cas où la nature réalise comme dans l'œuf, l'heureuse dissociation entre ces deux grandes fonctions de la vie cellulaire, il se produit sans doute, avant chaque division, une mobilisation partielle des réserves nutritives qui équivaut à l'absorption d'aliments extérieurs préalable aux divisions banales. Loin de vouloir exclure ces phénomènes d'ordre nutritif, il faut au contraire souhaiter qu'on les mette bientôt en pleine lumière. Il y a certes là des difficultés autrement grandes que celles que l'on a rencontrées en cytologie descriptive. Une fois réalisées, les découvertes fondamentales des pionniers de la Cytologie, des Flemming, des Van Beneden, des Boveri, leur généralisation dans le plan morphologique n'a pas rencontré d'obstacles énormes. Mais nous sommes arrivés à l'heure où il faudrait hisser nos conceptions sur le plan physiologique. Et pour cela il serait désirable de nous débarrasser de l'idée que tout ce qu'il y a d'important dans la cellule serait providentiellement colorable par l'hématoxyline, la safranine ou le violet de gentiane.

Je pense enfin qu'il faut attacher une attention toute spéciale à l'investigation du rôle des éléments chimiques communs à tous les milieux de prolifération cellulaire, c'est-à-dire aux éléments salins. Il est caractéristique que dans cette révision nous avons trouvé la trace de leur intervention—tout au moins dans les conditions expérimentales—pour ainsi dire à toutes les phases de la cytodiérèse: dans l'apparition des chromosomes, dans la dissolution de la membrane nucléaire, dans la polarisation de la figure mitotique, dans le déterminisme de la plasmodiérèse. L'impression est donc que l'on touche là à des facteurs d'importance vraiment générale, vraisemblablement physiologique, et qui se prêtent admirablement à l'analyse. Cependant, un autoréférat de Gellhorn (1927) vient de présenter sous un jour plutôt pessimiste un ensemble imposant de recherches personnelles relatives à l'action des sels sur la "perméabilité physiologique." S'il faut en croire cet auteur, les effets des sels sur la vie cellulaire et spécialement sur les œufs et les spermatozoïdes n'auraient aucun caractère de généralité et cela s'appliquerait surtout aux cations, dont l'activité physiologique est cependant si grande. C'est là une constatation troublante qui pourrait faire croire que l'analyse de l'activité cinétique par les effets des cations fait fausse route, parce qu'elle ne saurait se prêter à généralisation. Mais sans parler des nombreuses critiques de détail dont sont passibles les recherches d'ailleurs intéressantes de Gellhorn, critiques que Runnström a exposées dans un

article très documenté, on peut affirmer que ses conclusions sont au moins prématurées. D'une manière générale, on peut leur reprocher de mettre en jeu à côté des cations physiologiques, des agents tels que le lithium, le rubidium, le cæsium, qui sont pratiquement absents du milieu normal et dont l'effet toxique est certain. Et de plus, l'auteur traduit en termes de perméabilité, qui sous-entendent une interprétation physico-chimique relativement simple, des processus de complexité reconnue, tels que les mouvements des spermatozoïdes, la fécondation, la segmentation. Ses déductions sont donc basées sur des faits insuffisamment analysés. Rien d'étonnant, alors, à ce que des discordances apparentes se signalent chez les diverses espèces. Au contraire, si l'on a soin de se limiter à l'emploi des cations physiologiques, si l'on choisit un phénomène simple et qu'on en pousse l'analyse à fond, on est surpris de voir se dessiner, comme c'est le cas pour l'entrée en maturation, une unité d'effets vraiment frappante chez des espèces pourtant fort éloignées. Dès lors, la confiance renaît, et l'on se prend à espérer beaucoup de l'emploi de ces agents si commodes, dont on peut si bien comparer les effets *in vivo* et *in vitro*.

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THE PHYSIOLOGY OF OVARIAN ACTIVITY

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CONTENTS.

I. INTRODUCTION.

IN this review it is hoped to bring together the more important lines of work bearing on ovarian internal secretion. The literature on this subject, always vast, is growing rapidly, and while it is quite impossible to cover the whole subject, it is intended to give a fairly full account of the more recent work. A detailed account of the general ground-work of the subject is to be found in Marshall's *Physiology of Reproduction* (211), but a large amount of work has been done since the last edition of this book. A great deal of information, largely of a morphological nature, is to be found in Hammond's two books, *Reproduction in the Rabbit* (131), and *The Physiology of Reproduction in the Cow* (132). Lipschütz's book, *The Internal Secretions of the Sex Glands* (166), deals with the experimental basis (largely operative) of the conception of the gonads as endocrine organs, while the correlation of the gonads with the other endocrine organs has been exhaustively studied by Blair Bell (35).

As regards reviews, Marshall has given a synopsis of the subject up to 1923 (212), while Lipschütz (171) has recently discussed some of the factors governing the dynamics of the ovary. Allen and Doisy (16) in a still more recent review have dealt exhaustively with the properties and actions of the oestrus-producing hormone of the ovary, but scanty attention is paid to the whole subject of the corpus luteum, and to the luteal phase of the reproductive cycle. Reviews by Loeb (188) and Courrier (70), however, indicate the importance of the corpus luteum in the sexual cycle.

That the ovarian control of the growth and activity of the accessory organs is performed by means of internal secretions has long been surmised, and a complete discussion of this aspect of the subject with the fundamental evidence of ovariotomy and grafting experiments has been given by many authors (211).

The cyclic nature of ovarian activity has always complicated the study of its mechanism. Marshall originally postulated that the ovarian control of the reproductive processes was exerted by means of three internal secretions:

(1) One governing the development of the accessory organs and secondary sexual characters.

(2) One associated with oestrus.

(3) One (from the corpus luteum) controlling the changes of pregnancy.

This conception has since been accepted in essence by many authorities, including Loeb (188), and Courrier (70).

The comparatively recent preparation of oestrus-producing extracts in a tolerable concentration, together with the elaboration of an easy method of assay (Allen and Doisy and co-workers (10)), has given a tremendous impetus to the study of this phase of the cycle. The emphasis which is consequently being laid upon the follicular phase (to use Loeb's terminology) tends to obscure the importance of the corpus luteum in the control of the events which follow its development, and to overlook the pioneer work of Fraenkel (100, 109), Ancel and Bouin (20–27), Loeb (180) and Marshall on the functions of the luteal tissue. This tendency is exacerbated by the comparative failure, up to the present, to simulate the action of the corpus luteum by injection of extracts.

Allen and Doisy (16) justly remark that the best work up to 1920 stressed the importance of the corpus luteum, but it is equally true to say that the best work since that date has stressed the importance of the so-called follicular hormone. There can be little doubt that future work will redress the balance and vindicate Marshall's original conception.

II. GENERAL NATURE OF THE OESTROUS CYCLE.

The study of the oestrous cycle in its morphological and chronological aspects is a necessary preliminary to the study of ovarian endocrine activity. In recent years a great advance has been made in our knowledge of the details of the oestrous cycle in many animals, and particularly in Rodents. The study of the cycle in Primates is now being systematically undertaken, and holds promise of doing much to elucidate the problems of the human menstrual cycle.

The recent work on the oestrous cycle has been listed by Allen and Doisy [16], and only the more important will therefore be referred to here. Work up to 1921 has been adequately discussed by Marshall [211]. Since that date the cycle in the rat has been extensively studied by Long and Evans [208], while Allen [3] later applied the same technique to the mouse. Hartman [135] has extended his previous observations on the opossum, while the pig has been dealt with by Corner [61, 64] and McKenzie [209]. The cow has been investigated by Murphy and his co-workers [221-225], and in more detail by Hammond [132], who has also recorded extensive work on the rabbit. Corner [62] and Allen [8], working on *Macacus rhesus*, have reported results of great importance. Stockard and Papanicolaou [277, 278] have extended their original observations on the guinea-pig.

Less detailed investigations have been made on the mare (Seaborn [258]), and on the baboon (Gear [125]), while Engle [90] has reported some interesting observations on the Pacific Cetacea.

Allen and Doisy [16] in their recent paper have adequately reviewed the salient features of sexual periodicity and only a short account of the oestrous cycle in general is necessary here.

The cycle in the ovary of the unmated female consists essentially of the maturation of Graafian follicles, and the consequent production of corpora lutea. In some few mammals (rabbit [131], ferret [211]) ovulation only occurs after the preliminary stimulus of copulation, but in most cases ovulation is spontaneous at oestrus and is independent of copulation. In spontaneously ovulating animals oestrus may be followed very rapidly after a short dioestrous interval by a similar sequence of events, or, according to the species of animal, by a long phase of quiescence. In most of the ordinary laboratory animals the anoestrous period of quiescence is missing, and the ovarian cycle consists of the periodic maturation of groups of Graafian follicles with the intermediate development of corpora lutea. In the rat and mouse, for instance, under laboratory conditions this process in the unmated animal goes on indefinitely. The exact extent to which the development of the corpus luteum of ovulation in the unmated animal proceeds varies considerably according to the species. In the rat and the mouse the development is very slight (ovulation occurs every five days), and there is good reason for supposing that the corpora lutea of ovulation in these animals never becomes functional. In the guinea-pig, on the other hand, the cycle is of greater duration (16 days) and the corpora lutea of ovulation show a greater development and do become functional. It is known, for instance, that the removal of the corpora lutea shortly after ovulation results in expedition of the next oestrous period (Loeb [186]). A similar state of affairs is found in the cow, where the corpora lutea show considerable development, and are responsible for postponement of the next oestrus (Hammond [132]). In these animals, however, the corpora lutea of ovulation do not develop sufficiently far to cause any definite pregnancy-like changes in the accessory organs. In the dog, the ferret, the rabbit and *Dasyurus* [146] the corpora lutea of ovulation develop to such an extent that an actual simulation of pregnancy is brought about. This condition, known as pseudo-pregnancy, is the expression of the highest development

of the corpora lutea in the absence of conception. In certain circumstances, however, the development of the corpora lutea of ovulation in the rat and the mouse is more pronounced. Thus, after sterile copulation the next oestrous period is postponed for a period of some 12 days, more than twice as long as the normal dioestrous period, and a condition analogous with pseudo-pregnancy is found. Teleologically speaking, this is to allow time for the fertilised ova to become embedded if fertilisation has taken place. This postponement of the next oestrus is known to be due to the activity of the corpora lutea, and it may be said, therefore, that in the rat and the mouse the corpora lutea of ovulation only become functional (in the absence of pregnancy) after sterile copulation.

When ovulation is followed by pregnancy, the development of the corpora lutea becomes very considerable and correlated with this the period of gestation is characterised by complete absence of oestrus and ovulation. Furthermore, the degeneration of the corpora lutea at the end of pregnancy is probably the inciting factor in the onset of parturition. In the rat and the mouse ovulation follows immediately after parturition, and then, if lactation is taking place, no further ovulation is found for some three weeks. The oestrous cycle as a whole, therefore, may be divided into two phases: the follicular phase during which the Graafian follicles mature and ovulate, and the luteal phase, which includes pseudo-pregnancy and pregnancy, during which the corpora lutea dominate ovarian activity.

The indefinite nature of the symptoms of oestrus in most animals delayed very materially experimental work upon the causation of oestrus, and the sacrifice of an animal for each test of an extract made experimental progress relatively slow. Recently, however, the examination of intact animals by means of the vaginal smear technique has made possible the easy and rapid testing of any extract for oestrus-producing or oestrus-inhibiting properties, and has made possible rapid advance in this subject.

With the preparation of ovarian extracts capable of producing oestrous symptoms in ovariotomised animals, the initial problem of the causation of oestrus may be said to have been solved, but the further problems presented for solution are extraordinarily complicated. Roughly, these problems may be divided into three classes: (*a*) the actual nature, chemical constitution, and site of origin of the oestrus-producing hormone, (*b*) the mechanism whereby periodicity in effect is produced by hormone action, (*c*) the limitations of its action in the reproductive cycle considered as a whole. The progress made in solving these problems is discussed in Sections IV and V, while the action of the corpus luteum in throwing the cycle out of gear and in bringing about the characteristic changes of the luteal phase is dealt with in Section VI.

III. THE ATTAINMENT OF PUBERTY.

In any discussion of the factors responsible for the causation of puberty, it seems necessary to distinguish two phases in the attainment of puberty. In the first place, there is the slow and gradual development of the accessory organs of reproduction (the uterus and vagina, etc.) which occurs over a long period of

immaturity. Secondly, there is the climax to the attainment of puberty, the abrupt appearance of the first oestrus and ovulation.

There is clearly no reason to distinguish between the causative mechanism of this first oestrous period and that of any subsequent one. The underlying mechanism must be the same in each case, although at puberty the mechanism is operating for the first time. It has been very clearly demonstrated that injections of ovarian extracts will cause the immature uterus and vagina to show abruptly all the symptoms of oestrus (14, 99, 119), and to some extent one is justified in calling such an induced oestrous period "precocious puberty." It is, however, clearly no simulation of the slow and steady development which normally occurs before the time of normal puberty. All the work on the inducement of "precocious puberty" by the injection of oestrin has merely shown that the first oestrus is produced in the same way as any other. It seems, therefore, that the real problem of the attainment of puberty lies in the causation of the initial development of the uterus, and in why the first oestrus appears when it does.

Initial development of the accessory organs. As regards this problem, the factor concerned is known to be ovarian, and is furthermore known to be of a non-nervous nature (ovariotomy and grafting experiments). At the same time no successful attempt appears to have been made to simulate this gradual pre-pubertal development by the injection of ovarian extracts in the ovariotomised animal, and the final proof that the factor is endocrine in nature is therefore lacking.

It is, of course, possible that the oestrus-producing hormone is responsible for the initial development of the accessory organs. On this view the hormone is present before puberty in amounts insufficient to produce oestrus, but sufficient to result in uterine growth. Since there is nothing abrupt about the pre-pubertal growth of the uterus, this view assumes a radical change in its mode of action at puberty. Quite apart from such considerations, however, there is some reason to suppose that a separate ovarian factor (presumably hormonic) governs the development of the accessory organs. In support of this contention it is now possible to bring forward certain evidence derived from the results of work on the oestrus-producing hormone. This evidence relates to the hint of incompleteness which is found in the oestrous reaction of ovariotomised mice to injection. There is no doubt whatever that in many animals all the histological and physiological symptoms of oestrus can be produced in the absence of ovaries, but, in the experience of certain workers, ovariotomised animals will not copulate at an induced oestrous period. Parkes, Fielding and Brambell (244) report 92 induced oestrous periods in ovariotomised mice kept with males, and of these only seven were accompanied by copulation. Of these seven, three occurred in mice which were found to have regenerated ovarian tissue. So far, therefore, as these results go, copulation is emphatically not a normal occurrence during induced oestrus. Ovariotomised animals with regenerate ovarian tissue and normal mice with induced oestrus will copulate freely.

Other workers report copulation at induced oestrus: Allen and his collaborators (10), for instance, report that seven out of eleven rats copulated at an induced

oestrous period. The actual evidence, however, that copulation accompanies oestrus induced after ovariotomy is apparently meagre.

Further light on the incompleteness of induced oestrus has been brought forward by Asdell and Marshall[32] who found that in ovariotomised dogs the injection of the oestrus-producing hormone produced pro-oestrus symptoms without those characteristic of complete oestrus.

It seems possible, therefore, that the oestrus-producing hormone may not be capable of causing the complete symptoms of oestrus, and the missing factor may well be that responsible for control of the initial development of the accessory organs.

The determination of the first oestrus. In spite of a certain degree of individual variation, the age at first oestrus in each species is fairly constant, and this suggests that some definite mechanism exists for setting in motion the oestrous cycle. During the last few years much evidence has been accumulating which seems to show that the regulation of ovarian periodicity is external to the ovary. This evidence is dealt with in detail in Section IV (*b*), in considering the general mechanism of oestrous periodicity. It may be premised here, however, that this evidence is now almost conclusive, and it is necessary to admit that the regulation of the time of first oestrus may be of somatic origin.

Recent work on the anterior pituitary body suggests that the somatic control of ovarian periodicity may be hormonic. Smith and Engle[268, 272] have shown that the daily subcutaneous transplantation of anterior pituitary body to the young mouse will cause all the symptoms of oestrus to appear in about three days. This effect is not found in the ovariotomised young mouse, and the pituitary extract, therefore, acts through the ovary. In correlation with this it is found that the ovary has suddenly attained a state similar to that found in maturity and has begun to ovulate. These experiments are fully discussed in Section III (*b*). Since either male or female, young or mature anterior pituitaries are equally efficacious, it is clear that the experiments do not solve the question of what precipitates the first oestrus stimulus; they merely divert the crux of the problem from the ovary to the pituitary.

The production of precocious oestrus, therefore, either by ovarian or pituitary extracts, does not throw light on what is actually the causative factor in the liberation of the first oestrus-producing stimulus. It does, however, illuminate brilliantly the actual nature of oestrous production itself.

In the circumstances the work relating to the induction of precocious oestrus is considered with the work relating to the induction of oestrus in general.

IV. THE OESTRUS-PRODUCING HORMONE.

(*a*) PREPARATION.

Early extracts of ovary. The early workers on ovarian extracts appear to have endeavoured to prevent uterine atrophy following double ovariotomy[54, 149], by the injection of saline extracts. Later, Sonnenberg[273] attempted to produce oestrus by the injection of liquor folliculi. The first reported attempt to induce

oestrus by means of the injection of extracts appears to have been made by Adler [2], who claimed to have produced oestrous changes in virgin rabbits by injection of aqueous extracts of whole ovaries. Shortly afterwards, ovarian extracts made with organic solvents were used by Iscovesco [147, 148] who obtained a substance which caused rapid hypertrophy of the uteri of normal adult animals, the uteri produced being three or four times the weight of those of control animals. Fellner [99, 100] appears to have been the first to use the ovariotomised animal for the testing of ovarian extracts. This author produced oestrous symptoms in ovariotomised animals by the injection of alcoholic extracts of whole ovaries. Extraction of separate ovarian constituents was attempted by Okinschitz [228] in the following year. This author used ovariotomised rabbits as test animals and claimed that the subcutaneous injection of extracts of whole ovary and of liquor folliculi retarded the atrophy of the uterus after removal of the ovaries. Extracts of corpora lutea were not found to have this effect. At about the same time Seitz, Wintz and Fingerhut [261] claimed to have prepared from the corpus luteum two different substances, one of which promoted oestrus, while the other had an inhibiting action. That the oestrus-producing substance was present in the placenta as well as in the ovaries was shown by Herrmann and Frankel (*Eng. Pat.* 113, 1915), who, however, used immature rabbits as test animals. Frank and his collaborators [113-124] have published a series of papers since 1915, which reported observations roughly agreeing with those of Herrmann and Frankel. They also obtained an active substance from corpora lutea, the extracts being tested upon immature rabbits, and later upon rats. Aschner [30] obtained extracts from ovaries and placentae, which produced oestrous symptoms in ovariotomised guinea-pigs. Wintz [291] and Seaborn and Clampy [259] claim to have obtained positive results with injection of liquor folliculi.

Recent work on the extraction of the oestrus-producing hormone. By applying the vaginal smear technique to the testing of ovarian extracts, Allen and Doisy and their co-workers [10] were able to make great advances in the whole problem of ovarian internal secretion. Their extracts were made on much the same basis as those of earlier authors, but various elaborations were introduced. Allen and Doisy [10, 12] started with liquor folliculi from cow or pig ovaries as their raw material. The liquor folliculi was obtained by aspiration from the larger follicles with a hypodermic needle. The fluid was mixed with twice its volume of 95 per cent. alcohol and allowed to stand until the precipitated proteins had coagulated. The mixture was then filtered and the protein residue again extracted with alcohol to remove the fraction of the hormone adsorbed on to the proteins. The two alcoholic filtrates were then mixed and evaporated down to a watery residue. The residue was extracted with ether, this extract again being evaporated down. The residue from the ether was dried and extracted with acetone, the phosphatides being left behind. The acetone soluble fraction was again extracted with 95 per cent. alcohol for the purpose of eliminating some of the fat. Further purification of the extracts was obtained by taking up with methyl-alcohol and allowing to stand at 0° C. This procedure precipitated a great deal of the cholesterol. A very similar process, with certain necessary modifications in the initial stages, was used

for extraction of solid tissue. As a result of their initial investigations Allen and Doisy came to the conclusion that an extract could be obtained which would induce in the ovariotomised rat or mouse all the normal symptoms of oestrus. The main and essential source of this hormone Allen and Doisy considered to be the liquor folliculi. Very small yields from residual tissue were put down to the incomplete removal of the follicular fluid. They found that no oestrus-producing extract could be made from solid corpora lutea of lower mammals, but were later able to demonstrate its presence in certain cases in the human corpus luteum. Since this work of Allen and Doisy and their collaborators was begun, many authors have amplified and confirmed their main results. Among these may be mentioned the following: Frank and his co-workers (114–124), Courrier (67–73), Papanicolaou (229–231), Loewe and collaborators (193–207), Brouha and Simonnet (49–53), Dodds and collaborators (78, 79), Hart and collaborators (134), Zondek and Aschheim (294–297), Bugbee and Simond (55–57), Laqueur and collaborators (157–163), Lipschütz (170, 173, 174), Fellner (105, 106), Parkes and Bellerby (239–243).

The bulk of the work which has been done on the oestrus-producing hormone has followed the lines laid down by Allen and Doisy, and has been based on the assumption that the hormone is either of a fat nature, or else is closely associated with fats, and the methods of extraction have been based upon the supposition that fat solvents would give the best results. Comparatively recently, however, a variety of workers have endeavoured to obtain active extracts by a procedure assuming that the hormone could be obtained in a water-soluble form. Laqueur (162) tried a variety of means of precipitating the proteins, colloidal iron giving the best results. Liquor folliculi was diluted with four volumes of normal saline and colloidal iron then added. It was claimed that the hormone came through into the filtrate, which was quite clear. A large number of alternative procedures based on the same idea are given by Laqueur. Dodds (79) and his collaborators have recently published a description of a method by which they claim to be able to produce active picrate and then hydrochloride extracts. Zondek and Brahn (303), Loewe (194) and Glimm and Wadehn (126) have also claimed to have obtained active water-soluble preparations. These aqueous techniques seem, however, to be without confirmation up to the present.

Chemical properties. With the present doubt as to whether the hormone is actually fat soluble or water soluble, and with the admitted impurity of the present active extracts, it is clear that only a very rough approximation of its chemical properties can be given. Various organic solvents are definitely known to dissolve either the hormone itself or the fatty substances to which it is attached (alcohol acetone, ether, light petroleum, chloroform and benzene). According to the original work of Hermann, however, and also to much recent work, it is entirely insoluble in water. A list of more detailed, and therefore less certain, chemical properties is given in Allen and Doisy's (16) recent review, which should be consulted for further information. Jordan and Doisy (151) state that the hormone is destroyed by ultra-violet rays.

(b) ADMINISTRATION.

Oral administration. It appears to be generally agreed that preparations known to be active have no effect when given by mouth. Allen (10) reports five tests of administration by stomach tube with uniformly negative results. Bellerby (unpublished observations) found complete inactivity by mouth, while Loewe, Lange and Faure (196), though agreeing that the hormone is generally inactive by mouth, report that very large doses may cause a vaginal reaction. This question of the fate of the hormone in the alimentary canal is of some interest. Its inactivity by oral administration would naturally be put down to destruction by the digestive enzymes. Experimental work, however, has not so far confirmed this expectation (10). Furthermore, it is clear that the hormone is not destroyed by the degrees of acidity and alkalinity found in the gut. It is possible that the active substance passes through without being either destroyed or absorbed, but Bellerby (unpublished observations) was unable to detect any activity in extracts of faeces of mice to which large doses had been administered orally. Probably destruction takes place at some stage by digestive or bacterial action.

Injection. Subcutaneously the fat emulsions are absorbed reasonably well, provided that no large proportion of cholesterol is present and the hormone is quite active thus given. It was originally thought that intra-peritoneal administration might increase its efficiency, but no definite results seem to have been found. Allen and Doisy (10) were unable to demonstrate an increased activity intra-peritoneally, and the same result was obtained by Coward and Burn (74). Evans and Burr (95) even report an increased efficacy of subcutaneous when compared with intra-peritoneal administration. Intravenous injection does not appear to have been experimented with to any extent, and fat emulsions cannot be considered suitable material for such administration. Provided, however, that the emulsion is a reasonably fine one, and provided that only small amounts are used, it would seem that intravenous injection is a possible means of overcoming the undesirable local symptoms which are apt to attend subcutaneous injection. Certainly large amounts of fat emulsion can be given intravenously to certain animals without any adverse effects.

Attempts have been made to overcome the difficulty of maintaining a continuous supply of the hormone in the body by giving the dose in a series of small injections. Provided that the period over which injection is spread is not too great, it has been shown that no larger total amounts are required than when the whole dose is administered at one injection.

(c) ASSAY: DOSAGE.

The rabbit uterus. The early workers (2, 147, 148) with ovarian extracts used immature animals, usually the rabbit, as test animals, and considered an abrupt hypertrophy of the uterus as a positive result. Later on, ovariotomised rabbits were used for the same purpose. While the ovariotomised animal was clearly an advance on the immature one, neither can be said to have been ideal test animals, owing to

(*a*) the uncertainty of the nature of oestrous symptoms in the rabbit uterus, (*b*) the necessity for autopsy at every test, and (*c*) the lack of an end-point in a positive reaction. (See Uhlmann (282), for a discussion of this and other techniques.)

The vaginal smear technique. The discovery in 1917 by Stockard and Papanicolaou (277) in the guinea-pig, that vaginal changes coincided with the uterine and ovarian changes of the oestrous cycle, and, further, that such vaginal changes could be detected by examination of the vaginal contents, paved the way for the present ease with which ovarian extracts can be tested. This technique was extended to the rat by Long and Evans in their classic work on the oestrous cycle in the rat, and was later worked out for the mouse by Allen. The period of oestrus is characterised by an intense cornification of the vaginal epithelium and as the cornified cells are sloughed they may be detected in the vaginal contents. The post-oestrous phase is characterised by a very marked infiltration of leucocytes among the cornified cells, while during dioestrus the vaginal smear consists of varying proportions of leucocytes and nucleated epithelium. At the onset of pro-oestrus the leucocytes disappear from the smear, which is then composed purely of nucleated epithelium. The methods of removing the contents of the vagina and of mounting them for microscopic examination vary in small details, and individual authors may be consulted for accounts of their own particular methods. A more serious lack of uniformity in the application of the vaginal smear technique is that whereas most workers consider the full appearance of oestrous symptoms—including cornification —to be necessary before a reaction can be called positive, others (Laqueur (162), Lipschütz et al. (174)) have used the appearance of the pro-oestrous smear as indicating a positive result, while Loewe (202) used a method based on the proportions of the different types of cells. The use of the pro-oestrous smear can be supported on the grounds that a smaller amount is required to produce it and, therefore, that greater accuracy in testing can be attained. The pro-oestrous smear is, however, much more likely to be confused with variations of the operative dioestrous smear than is the clear-cut oestrous smear. For this reason its use is to be deprecated, especially, as Allen and Doisy (16) remark, in new qualitative work.

In cases where any doubt exists the vaginal smear diagnosis may be checked by autopsy and examination of the uterus.

Any attempt to test ovarian extracts almost presupposes the previous removal of the ovaries in the test animal. Use might be made of the anoestrous period in animals (such as the dog) where it occurs. The danger of indirect action through the ovary is, however, always present.

Other criteria of oestrus-producing activity. Various other means have been suggested for testing extracts for oestrus-producing activity. Swelling and hyperaemia of the areas around the external genitalia are a marked feature of oestrus in certain animals (dog, monkey), but the comparative absence of such symptoms in convenient laboratory animals, and their lack of a definite end-point renders them highly unsuitable in assaying extracts and they appear to have been used but little (100, 141, 273).

The use of the mammary glands as test objects in the assay extracts is, of course, essential if extracts are being tested for the induction of mammary growth. However, unless and until mammary growth is demonstrated to be a feature of oestrus in the animal in question (as in the opossum (139)), or is demonstrated to be produced generally by oestrus active extracts (which has yet to be shown), the consideration of mammary growth as a criterion of oestrus-producing activity is clearly illegitimate. In any case the induction of mammary growth cannot be considered specific for oestrus in the same way as cornification of the vagina is.

From time to time entirely different methods of assaying the activity of oestrus-producing extract have been suggested. Frank and his co-workers (118), Seckinger (260) and Brouha and Simonnet (52) have suggested that the effect of extracts on the contraction of the isolated uterus could be used as a criterion of its oestrus-producing effect. Up to date, however, no methods which have been experimented upon appear to give the easy and reliable results of the vaginal smear technique.

Time of testing after operation. It might be supposed that the time elapsing between the operation of ovariotomy and the injection would affect the amount required to produce a positive result. It seems probable in fact that after a prolonged period the sensitivity of the uterus would diminish, especially if no intermediate injections had been made. Constant injections might keep the uterus up to its normal activity. Allen and his collaborators (10) report that the previous duration of the operative dioestrus has very little effect upon the effectiveness of injection. Their longest time without induced oestrus was 15 weeks, and no decrease in sensitivity was observed at the end of this period. Coward and Burn's (74) results indicate the same thing, though in this case the animals were injected at regular intervals.

Ovarian regeneration. Since the basis of testing ovarian extracts is the absence of the ovary, the possible presence of ovarian tissue in ovariotomised animals is of primary importance. This presence may occur in one or both of two ways: (*a*) accessory ovaries may have been present before ovariotomy and have escaped removal, and (*b*) even after the complete removal of all ovarian tissue at the time of operation, new tissue may regenerate at varying periods after the operation.

Accessory ovaries are very rare in rodents. The writer has dissected many thousands of female mice, but only in one case has a third ovary been detected. In this instance the accessory ovary occupied a common capsule with the normal right ovary, and would therefore have been removed at ovariotomy. Even where their occurrence is more common, it should be possible to detect the presence of third ovaries by the failure of double ovariotomy to produce dioestrus.

The regeneration of ovarian tissue is, however, a more serious complication. This phenomenon has been noted by Castle and Phillips (58) and Davenport (77) and referred to by Frank and Goldberger (121). The apparent improbability that ovarian tissue could regenerate *de novo* after complete double ovariotomy has always fostered the suspicion (especially in clinical cases) that the original ovaries have been incompletely removed. Parkes, Fielding and Brambell (244), however, have shown quite definitely by seriation of the ovaries removed at operation that even the complete elimination of all tissue which can reasonably be called ovarian may be

followed by the appearance at some later date of ovarian tissue containing follicles and corpora lutea. These authors came to the following conclusions:

(*a*) That the complete removal of ovary, capsule, and hilum is followed by regeneration in about 10 per cent. of cases.

(*b*) That such regeneration may occur up to many months after the operation.

(*c*) That regeneration can best be detected by the spontaneous appearance of oestrus, and that adequate observations for such spontaneous oestrus should be made between each experimental injection.

(*d*) That any anomalous test result should be repeated on another animal and the first one examined for ovarian regeneration.

The unit. The unit of the hormone has generally been considered to be the least amount which would cause the production of full oestrous symptoms in the ovariotomised animal, and practically all the work so far done on the standardisation of extracts has proceeded on this basis. In practice this technique meant that a certain amount would be given to one or more ovariotomised test animals, and then, according to its positive and negative reaction, lesser or greater amounts would subsequently be tested. By this means the least amount required for a positive result could be arrived at. Recently, however, it has been shown by Coward and Burn[74] that in large batches of animals the individual variation in response to a given amount may be so great as to invalidate this method. These authors found that whereas 2·5 mg. of an extract would produce oestrus in about 10 per cent. of ovariotomised rats, 17·5 mg. would only produce oestrus in a little over 80 per cent. Intermediate values gave a typical S-shaped curve, which has been previously found as typical of the response of batches of animals to various drugs and poisons. As a result of this work Coward and Burn suggest that the animal unit shall be defined as the amount necessary to bring 50 per cent. of ovariotomised animals into oestrus.

These methods of arriving at the strength of extracts both involve considerable labour, and a correlation which has recently been found by Tuisk[281] and Parkes and Bellerby[241] suggests a possible short cut. Parkes and Bellerby found that a linear relation existed between the dose given and the duration of the resulting oestrous symptoms, and it is possible that such a relationship could be used to calculate the unitage after a single positive injection.

Individual variation in response is, of course, the difficulty in the application of such an experimental finding, but a standard table worked out from animals of a reasonably homogeneous colony used in conjunction with tests on animals of the same colony should reduce the effect of individual variation to a reasonable level.

The relation of the unit of the hormone in various animals, namely, the relative amounts required to bring various species of ovariotomised animals into oestrus, appears to have received very little attention. Allen and Doisy[16] state that two to four times the amount required for a mouse is required for a rat, but Coward and Burn[74] found that their curves of percentage animals brought into oestrus by varying doses were identical for both rats and mice. Bugbee and Simond[55] state that the rat dose is two to eight times that required for a mouse. As regards

other animals, little evidence appears to be available, but provisionally, it would seem possible to say that the unit ratios do not follow a weight for weight basis. Pratt and Allen (246) claim to have produced clinical effects with very small doses.

Working on the principles outlined above, many authors have stated the purity of their preparations in terms of the weight of the unit. Some of the results are given below in Table I.

Table I. *Weight of the unit of Oestrin.*

Source of extract	Weight of unit (mg.) R.U. or M.U.	Method of assay	Authority
Liquor folliculi, max. purity	0·13 (R.U.)	Smear technique	Doisy, Ralls, Allen and Johnston (82)
Liquor folliculi, min. purity	19·5 (R.U.)	,,	,, ,, ,,
Whole ovaries	64·0 (R.U.)	,,	,, ,, ,,
Placenta, human	25·0 (R.U.)	,,	,, ,, ,,
Unstated	10·0 R.U. or M.U.	,,	Coward and Burn (74)
,,	2·25	,,	Frank and Gustavson (123)
Liquor folliculi	0·00002 M.U.	,,	Lipschütz et al. (174)
Liquor folliculi:			
Cow	12·1–2·61 (M.U.)	,,	Parkes and Bellerby (239)
Pig	21·5–14·2 (M.U.)	,,	,, ,,
Horse	9·4 (M.U.)	,,	,, ,,
Whole mature ovaries:			
Cow	6·5	,,	,, ,,
Pig	12·6	,,	,, ,,
Residual tissue:			
Cow	16·9–9·0 (M.U.)	,,	,, ,,
Pig	35·0–10·5 (M.U.)	,,	,, ,,
Horse	19·6 (M.U.)	,,	,, ,,
Placenta:			
Human	27·7–3·83 (M.U.)	,,	Parkes and Bellerby (242)
Cow	19·2–1·9 (M.U.)	,,	,, ,,
Sheep	21·7–6·25 (M.U.)	,,	,, ,,

Liquor folliculi would therefore appear to give the purest extracts, but the enormous variations in the purity of extracts obtained by different workers, and even in different preparations by the same workers, make an accurate grading of the sources of supply impossible.

Nomenclature. To call the oestrus-producing hormone of the ovary "the ovarian hormone" is to pre-suggest that only one exists. The term "follicular hormone" is only of relevance in so far as it shows the essential connection with the follicular phase of the cycle. The term "folliculine" proposed by Courrier (69) suffers from the same disadvantage. "Menform" and "thelykinin" have been proposed by Laqueur and Loewe (204) respectively. The use of a name for descriptive purposes is clearly advantageous, and since the substance in question has not adequately been shown to produce any features other than those characteristic of oestrus, the name "oestrin" (Parkes and Bellerby (239)) will be used throughout this review.

(d) DISTRIBUTION AND YIELD.

Distribution. Liquor folliculi has mainly been used as the original source of the hormone and the follicle has been regarded as the essential site of origin. The hormone, however, had a far wider distribution than this. The stromal tissue of the ovary was known to contain a certain amount, and this amount has recently been shown to be greater than can reasonably be accounted for by the presence of small follicles[139]. The corpus luteum has variously been said to contain the hormone and not to contain it. Iscovesco[148], Seitz, Wintz and Fingerhut[261], Okinschitz[228], Herrmann[41], Frank and Gustavson[123], Glimm and Wadehn[126] have all claimed to have produced oestrous extracts from corpus luteum. Allen and Doisy[10], however, failed to do so with material from the cow and the pig, while Johnston and Gould[150] also failed with pig corpora lutea. Allen and Doisy[15] did, however, find activity in preparations of human corpora lutea. Parkes and Bellerby[243] found that in the cow corpus luteum the hormone appeared to be restricted to the fluid content of hollow specimens which amount to some 25 per cent. of the corpora lutea in this species. They failed to obtain oestrus-producing extracts from solid corpora lutea. This work seems to offer an explanation of the contradictory result obtained by previous workers on the corpus luteum and, since the fluid of hollow corpora lutea is a derivative of the liquor folliculi, seems to show that the corpus luteum tissue itself does not elaborate the hormone. In view of the functional correlation of the corpus luteum with the absence of oestrus it would certainly seem anomalous for an oestrus-producing substance to be elaborated by this organ. It may thus be said that the ovaries, including the liquor folliculi of all mammals which have been examined, contain this hormone, while the corpora lutea contain it doubtfully or incidentally. It has been known for a long time that comparatively large amounts of oestrus-producing substance could be obtained from placentae, and this has been confirmed by many investigators[12, 13, 82, 123, 222]. The foetal membranes and even liquor amnii have been found to contain the hormone, but extracts of embryos have given uniformly negative results. Various workers have claimed that the hormone may be detected in blood (Loewe[193], Fels[107], Smith[267]), and Frank and his collaborators[116, 120–122] even go so far as to describe variations in the content of the blood according to the stage of the menstrual cycle, and even according to intersexual conditions of the gonads. Loewe claims to have obtained positive results from urine. Various control materials, the liver, pancreas, muscle have been found to give negative results. Fellner[104], Laqueur, and Robinson and Zondek[255] have claimed that active extracts may be obtained from testes, but this has been contradicted by Allen and Doisy and their co-workers[82]. Little is known about the non-mammalian distribution of this hormone. Fellner[106] has claimed to have detected activity in the eggs of hens and fish, but this again has been contradicted by Allen and Doisy. Loewe and his co-workers[204] claim to have detected oestrin in various plants.

Yields. The variety of methods employed for the extraction of the hormone, together with the variation in the methods of assay, invalidate any strict comparison

of the yields reported by different authors. The following table, however, sums up some of the data put forward by various authors.

Table II. *Yields of oestrin from various sources.*

Source	Yield per kilo	Observer
Whole ovaries:		
Cow	293 M.U.	Parkes and Bellerby (239)
„ (immature)	73–350 M.U.	„ „
Pig	219 M.U.	
„	120 R.U.	Doisy, Ralls, Allen and Johnston (82)
„ (immature)	166–273 M.U.	Parkes and Bellerby (239)
Sheep (anoestrous)	203 M.U.	„ „
Liquor folliculi:		
Cow	37–788 M.U.	„ „
Pig	23–75 M.U.	„ „
Horse	113 M.U.	„ „
Pig	878 R.U.	Ralls, Jordan and Doisy (250)
„	600–1600 M.U.	Laqueur, Hart, De Jough and Wijsenbeek (163)
„	167 R.U.	Dickens, Dodds and Wright (78)
Human	433–7000 R.U.	Allen, Pratt and Doisy (19)
Residual ovarian tissue:		
Cow	150–326 M.U.	Parkes and Bellerby (239)
„		Dickens, Dodds and Wright (78)
Pig	227–865 M.U.	Parkes and Bellerby (239)
Horse	27 M.U.	„ „
Corpora lutea:		
Human	3700 R.U.	Allen, Pratt and Doisy (19)
Pig	>8 but <25 R.U.	Allen and Doisy (16)
Cow (unsorted)	16	Parkes and Bellerby (243)
„ (solid)	No yield	„ „
„ (tissue of hollow corpora)	11 M.U.	„ „
„ (fluid of hollow corpora)	184 M.U.	„ „
Placenta:		
Human	192–2123 M.U.	Parkes and Bellerby (242)
„	400–700 R.U.	Doisy, Ralls, Allen and Johnston (82)
Cow	192–2123 M.U.	Parkes and Bellerby (242)
Sheep	183–308 M.U.	„ „
Cow (maternal)	203–3200 M.U.	„ „
„ (foetal)	143–782 M.U.	„ „

(e) MODE OF ACTION.

The means whereby the cyclic action of the oestrous hormone is regulated will not be considered here (see Section v), but it is necessary to discuss certain aspects of its local action.

Reaction time. Allen and his co-workers (10) originally reported that the initial changes in the vaginal smear of the ovariotomised animal could be detected 40–48 hours after injection, but these changes are, of course, preceded by active mitotic cell division in the uterine and vaginal epithelium. That about two days is the normal reaction time for smear changes has been amply confirmed by later workers. Courrier (72) and Brambell and Parkes (48) have in addition shown that the reaction time is not affected by the size of dose given. Thus 20 mouse units produce no quicker result than 2 M.U. The reaction time does not appear to vary with the

age of the animal. Immature mice and rats show oestrous symptoms as soon after injection as do adults.

Effect of continuous injection. It was soon found that prolonged oestrous symptoms could be produced by continuous injection. Allen(10), however, reported that a small infiltration of leucocytes was liable to occur during the periods of continuous cornified smears. This was explained on the grounds that the uneven absorption of the emulsions made it difficult to keep up a continuous supply of the hormone. Brouha and Simonnet(49) claim that it is not possible to prolong oestrous symptoms for more than four to five days, and these authors consider that this is evidence in favour of the view that the vaginal cycle is regulated by a cyclic sensitivity of the organ to the hormone. Frank, Kingery and Gustavson(119) found it possible to prolong cornification for eight to nine days, and similar results were later reported by Tuisk(281). Parkes and Bellerby(241) found that up to 13 days cornification could be produced by one injection (17 M.U.). The well-known cases of persistent oestrus in the normal untreated animal(71, 211) are analogous evidence. It seems possible to conclude, therefore, with Tuisk, "that we shall always find a prolonged oestrus if in any way the threshold concentration of follicular hormone is maintained in the blood." Further, it seems highly improbable that the vagina has any periodic sensitivity to the hormone.

Action on male. Various authors have investigated the effect of the ovarian follicular hormone on the male. Adverse effects upon the testes have been reported by Herrmann and Stein(144), Fellner(104), Gould and Doisy(16) and Laqueur(158). Adverse effects were also produced by injections of other lipoid substances (even testis lipoids) and it is most probable that the effects of the oestrous hormone are not specific. Definitely negative results have been reported by Bugbee and Simond(57).

Effect on growth and activity. Gonadectomised animals of both sexes attain greater weights than normals. Wang, Richter and Guttemacher(286) have demonstrated that this extra weight is lost in the castrated male grafted with ovarian tissue (see also(274)). Bugbee and Simond(57) claim to have extended these results by showing that injection of the oestrous hormone brings about a reduction in weight in both normal and gonadectomised animals of each sex.

Slonaker(263, 264) and Wang, Richter and Guttemacher(286) have shown that the voluntary activity of rats has a periodic rise and fall which is correlated with the oestrous cycle. Maximum activity is found at the time of oestrus. This cyclic activity ceases after ovariotomy, restarts after ovarian grafting (Wang, Richter and Guttemacher(286)) and can be caused in the ovariotomised animal by periodic injection of the oestrous hormone (Bugbee and Simond(57)); continuous injection leads to increased activity so long as injection is kept up.

Clinical use. The doubt as to the precise limitations of the functions of the "follicular" hormone makes it difficult to discuss the part which it might play in the restoration of aberrant reproductive function. The one action conclusively known to be performed by oestrin, *i.e.* the production of oestrous symptoms in the accessory organs, would seem to be of little value in itself in either veterinary

or human therapy. If the hypothesis that follicle stimulating action is performed by oestrin is substantiated, a much larger field is of course opened up for therapeutical work, since by this means the indirect production of the luteal phase could be brought about.

Murphy and his co-workers (224, 225) have described the use of ovarian extracts in veterinary practice in the treatment of aberrations of the reproductive function. The dosage appears to have been very small (no measure of any kind of the amount given is recorded) and the hopelessly inconclusive results are of little value.

Pratt and Allen (246) investigated the effects of small doses of the oestrus-producing hormone (one R.U. per day for varying periods) in the human in cases of amenorrhoea and menopause (artificial and natural). In some instances some uterine hypertrophy was diagnosed, but menstruation was not produced.

The clinical use of the hormone has also been reported on by Hermann (143), Zondek (292), Seitz, Wintz and Fingerhut (261).

Administration during senescence. Laqueur (158) and Steinach, Heinlein and Weisner (275) have reported the induction of oestrous symptoms in rats and mice during senescence when the normal cycle had definitely ceased. In a few instances the re-started cycle continued for a short time without further injections.

Specificity of reaction. It has been shown pretty definitely by means of extracts of a variety of tissues that the reaction of the vagina, etc. to oestrin is quite specific. In addition, histamine (Levin (164)) and the sexual stimulants yohimbin (Loewe (206)) and cocaine (Parkes, unpublished observations) have been found to produce negative results when tested for oestrus-producing properties on the ovariotomised animals.

(f) SITE OF ORIGIN AND SCOPE OF FUNCTION.

Site of origin. Since the maturing follicle is essentially associated with oestrus in the normal animal, the source of the oestrus-producing stimulus was long considered to be the follicle during the later stages of its growth. When the oestrus-producing hormone was discovered, and particularly when it was found to be abundant in the liquor folliculi, this view appeared to be confirmed. Recent work, however, has shown that the hormone is to be found in situations where it cannot reasonably be supposed to be elaborated (*i.e.* amniotic fluid, foetal membranes), and it is necessary to admit, therefore, that its occurrence in a particular site is not indisputable evidence of its elaboration there.

Since it is produced in the body of the non-pregnant animal the placenta is clearly not the initial site of elaboration. The corpus luteum, also, is clearly not essential, because the first oestrous period is produced in the absence of corpora lutea. The X-ray sterilisation work (Parkes (234–237)) discussed in Section v (*a*), makes it evident that the follicle, even if the essential organ in the normal animal, can be superseded in certain cases by non-cyclic tissue.

Scope of function. Any hypothesis as to the site of origin of the hormone is, however, influenced by the view taken as to its scope of function. One school of thought maintains that the oestrous hormone is the one and only ovarian hormone and is responsible both for the occurrence of oestrus and also for the changes

occurring during pregnancy. This view, of necessity, assumes that the corpus luteum elaborates the oestrus-producing hormone, at least for a time, and that the placenta is also an actual site of origin. Frank and Gustavson [123] have in fact applied this term "gestational gland" to the follicle, corpus luteum and placenta as the successive elaborators of the "female sex hormone." Allen [8] states: "That the human corpus luteum may contain considerable amounts of this active material has been demonstrated, but its utilisation is clearly a continuation of, or supplementary to, the hormonal function of the follicle. The continuous availability of this material from the placenta, present in amounts increasing with placental growth with the advance of gestation, is the most logical explanation of the growth of the uterus and mammary glands, and the absence of menstruation during pregnancy."

This view is of course in direct opposition to the great mass of work, begun by Fraenkel [109, 110], and extended by Ancel and Bouin [20-27], Loeb [180] and Marshall [211], which shows that the corpus luteum itself is responsible for the phenomena characteristic of the post-ovulative or luteal phase of the cycle. Allen [10] entirely failed, in common with other workers, to obtain the "follicular" hormone from corpora lutea other than human, and his supposition, as outlined above, reduces the corpus luteum to a mere histological ornament.

The evidence that the corpus luteum is responsible for the typical post-oestrous changes is considered fully in Section VI, but in this Section, dealing with the oestrus-producing hormone, it remains to consider what evidence, if any, is to be found that these changes can be brought about by the oestrus-producing hormone. Four functions have been assigned to the corpus luteum:

(*a*) Inhibition of oestrus and ovulation.

(*b*) Preparation of the uterus for the reception of the fertilised ovum.

(*c*) Maintenance of pregnancy.

(*d*) Development of the mammary glands.

The one indubitable function of oestrin is to bring about the oestrous changes in the accessory organs, and it is not probable, therefore, that it can be associated with the inhibition of oestrus. Similarly, injection of the "follicular" hormone during pregnancy has been shown to cause abortion [240, 266], and therefore it can scarcely be the ovarian factor responsible for the maintenance of pregnancy.

Oestrin and the post-oestrous uterus. The question of what part, if any, is played by the "follicular" hormone of the ovary in the preparation of the uterus for the reception of the ovum and in the production of post-ovulative phenomena generally is much more complicated. In animals such as the mouse and the rat the uterine changes which are characteristic of oestrus (and which are produced in the ovariotomised animal by the injection of oestrin) pass off before implantation, and have clearly no relation thereto. The continued injection of oestrin in the ovariotomised animal leads to the continued appearance of oestrous symptoms and not to any kind of post-ovulative or pseudo-pregnant phenomena. In the normal mouse and rat the injection of oestrin after copulation, leading to a return of oestrous symptoms,

results in regular failure to conceive. In the rat and the mouse, therefore, it is justifiable to say that the "follicular" hormone is not concerned with the regulation of post-oestrous changes and that the experimental prolongation of its activity is in fact directly antagonistic to them.

In the guinea-pig (Loeb (183)) and the cow (132) the antagonism between the follicular phase and the luteal phase has been well demonstrated and antagonistic phases can hardly be produced by the same hormone.

The problem really turns on whether or not oestrin is elaborated by the corpus luteum, and the view of Frank and his collaborators, that oestrin is the one ovarian hormone, regulating the sexual cycle in all of its phases, is based largely upon this supposition. Apart from the inherent improbability that an organ whose development is always associated with the absence of oestrus should elaborate the oestrus-producing hormone, other workers, including Allen and Doisy (10) and Johnston and Gould (150), have emphatically shown that oestrin is not present in the cow corpus luteum (Frank's material). In addition Parkes and Bellerby (243) have demonstrated fairly clearly that oestrin may be contained in the fluid centre of hollow corpora lutea, but not in corpus luteum tissue.

"In corpora lutea of the cow, oestrin is found only in the fluid contained in the cavities of hollow specimens, and since this fluid appears to be homologous with liquor folliculi it may be supposed to obtain its oestrin from the same source as does the latter. The absence of oestrin from solid corpora lutea seems to dispose of the suggestion that it is elaborated by the corpus luteum." (Parkes and Bellerby (243).)

In the rabbit the prolonged period of oestrus which is found in the absence of copulation produces none of the changes which are characteristic of pseudo-pregnancy, and this animal provides an excellent denial of the hypothesis that the same hormone is responsible for the production of oestrus, and afterwards of the post-oestrous phase, and that ovulation merely transfers the elaboration of the "follicular" hormone from the follicle to the corpus luteum. Long and Evans (208) have shown that the onset of the next oestrus is fatal to the existence of placento-mata.

In a recent paper Allen (8) has described remarkable experiments on *Macacus thesus*. His results may be summed up as follows:

(1) Ovariotomy before menstruation is due leads to its premature appearance. Injury to large follicles leads to the same result.

(2) Injection of the ovariotomised female with the follicular hormone results in the rapid coloration of the sexual skin. This reaction equals in intensity the phenomenon as found in the normal animal.

(3) Cessation of injection is followed by the appearance of menstruation. This reaction, however, appears from the protocols to be less regular and much less intense than is found in the normal animal.

Allen explains these results on the grounds that the follicular hormone is responsible for the pre-menstrual congestion of the uterus, and that its secretion is carried on after the rupture of the follicle (for a short time) by the corpus luteum[1]

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[1] Allen (15) found that, unlike cow and pig corpora lutea, the human corpora lutea contained oestrin.

and afterwards (in the event of pregnancy) by the placenta. If the production of the "follicular" hormone and the consequent stimulation of the uterus is stopped prematurely by

(*a*) the atrophy of the corpus luteum, and the failure of placental tissue to appear (in failure to conceive),

(*b*) the experimental damage to large follicles or double ovariotomy,

(*c*) the cessation of injection in the ovariotomised animal,

then uterine retrogression resulting in menstruation sets in.

Allen's explanation of the experimental results assumes the continued activity of oestrin from the beginning of the follicular phase to the end of pregnancy. The whole explanation is, however, complicated by the doubt as to the demarcation of the phases of the primate menstrual cycle. The ordinary gynaecological view is that the pre-menstrual congestion is purely of luteal origin, and that menstruation is practically the removal of useless decidua. This view is supported by the fact that prevention of corpus luteum formation in the human stops the pre-menstrual congestion required for menstruation. Marshall [211], however, holds the view that menstruation is at least partly pro-oestrous in nature. This view is supported by the fact that in certain lower mammals haemorrhage occurs during pro-oestrus. So far as *Macacus* is concerned, Marshall's supposition would appear to have been made almost certain by the observations of both Corner [62] and Allen [8] who have shown that menstruation may take place in untreated animals which have never ovulated and which, therefore, are entirely devoid of corpora lutea. Corner [63] has just published a most important paper showing that, although the non-ovulating *Macacus* does menstruate, the typical congestion of the uterus which precedes menstruation in the ovulating animal is absent, and the degree of haemorrhage is less severe.

This result shows that in *Macacus*, at any rate, menstruation is not entirely dependent upon the corpus luteum, and the simulation of menstruation in the injected animal does not necessarily mean replacement of the corpus luteum by the injections. All Allen's experimental results are, in fact, in keeping with the supposition that his injected animals only showed that part of the menstrual cycle which is independent of the corpus luteum and which is found in the non-ovulating *Macacus*. Since Corner has made it evident that the non-ovulating animal fails to exhibit the complete menstrual phenomena, the uncertainty of appearance and the scantiness of the menstruation of Allen's ovariotomised injected animals is most suggestive of such a comparison.

The reddening of the "sexual skin" appears from Allen's normal protocols to be associated with ovulation in the normal *Macacus*, and its production in the ovariotomised animal by the hormone associated with that stage of the cycle, though of great interest, is not therefore surprising.

Oestrin and the mammary glands. The castrated male guinea-pig with an ovarian graft assumes a condition which is known as hyperfeminisation, and which has been likened (just why it is difficult to see) by Lipschütz [172] to prolonged oestrus. The ovarian graft loses its cyclic nature, the follicles become cystic and no corpora lutea

are formed (33, 168). The male mammae undergo development and actual secretion of milk may result. This fact suggests that the presence of luteal tissue may not be essential for the development of the glands (see Section VI), and the idea that development of the mammary glands during pregnancy can be due (as supposed by Allen (8)) to the continued secretion and activity of oestrin is a possibility of great importance. Various authors have reported that certain effects are produced in the mammary glands by oestrin injection. Very early in the work on ovarian extracts Herrmann (141) reported hypertrophy of the mammary tissue in the injected rabbit, a result confirmed by Fellner (100) and Ancel and Bouin (27). Aschner (30) reported the same in the guinea-pig. Frank and Rosenbloom (124) and Laqueur report similar results. In the opossum the reaction of the mammary tissue to oestrin is very marked (Hartman and co-workers (17, 139)). Superficially, these findings also appear to be evidence against the mass of data (Section VI) which seem to show that the corpus luteum is responsible for the development of the mammae. Actually, however, this is not necessarily the correct interpretation. In several species it has been shown that a certain degree of mammary proliferation may appear at the time of oestrus and is therefore just as much an oestrous symptom as are the more striking changes in the other accessory organs. Thus, in the guinea-pig, Loeb and Hesselberg (190) have shown that proliferation in the mammary tissue takes place at oestrus, and that this proliferation is much less in amount and is separated by a period of rest from the subsequent proliferation which he showed to be directly under the influence of the corpus luteum. Similarly, Ancel and Bouin (27) and Vintemberger (283) distinguish very definitely in the rabbit between the slight mammary proliferation characteristic of oestrus (which they showed can be brought about after ovariotomy by injection of oestrin) and the extensive hypertrophy which is characteristic of pregnancy and pseudo-pregnancy, and which is not found during even the most prolonged oestrus and cannot be caused by oestrin injection. The description of the pre-pubertal growth of the mammary gland of the rat given by Myers (226) suggests that in this animal also a small proliferation takes place at oestrus.

The mammary development caused by the injection of oestrin in the opossum is clearly also of an oestrous nature. In this animal very extensive proliferation takes place at the time of oestrus (Hartman (135)), and it appears to be this phase which is brought about by oestrin injection.

It is necessary, therefore, to distinguish between (a) mammary development of oestrus, which is as definite an oestrous change as is cornification of the vagina, and which can, therefore, be induced in the ovariotomised animal by oestrin, (b) mammary development of pregnancy. The absolute failure to produce the latter by prolonged oestrin injection and its absence during prolonged oestrus show fairly conclusively that the former is not just turned into the latter by the continued production of oestrin by the corpus luteum after ovulation. The corpus luteum of pregnancy or pseudo-pregnancy clearly initiates an altogether new phase of growth in the mammary gland.

Significance of oestrin in the placenta. One of the real difficulties in supposing

that oestrin is not a vital factor in the changes characteristic of pregnancy is to explain on other grounds its very abundant occurrence in the placenta. It appears to be this difficulty which has led Allen and his co-workers to suppose that the whole of the changes characteristic of both oestrus and the luteal phase of the cycle are regulated by oestrin. It is, however, almost as difficult to assume that the placenta elaborates oestrin as to assume that it does not. The only evidence in favour of the hypothesis is its abundance in the organ, and, as mentioned above, that is far from conclusive. On the other hand, it is known (Parkes and Bellerby [240], Smith [266]) that injection of oestrin during pregnancy leads to abortion, and its secretion by the placenta would be virtually an attempt at suicide. Also, weight for weight, the placenta contains as much oestrin as the ovaries, and as the placenta weighs anything about 500 times as much as the ovaries, it contains about 500 times as much oestrin. If the placenta elaborates oestrin, and at the same rate as the ovaries, this means that the placenta is producing 500 times as much as the ovaries. And yet no symptoms of oestrus occur during pregnancy, and oestrin injection during pregnancy is fatal.

An alternative hypothesis has been expressed as follows: "If, however, it is assumed as a tentative working basis that oestrin is not elaborated by the placenta, it is necessary to account for its occurrence there in far greater amounts than it is found in any other organ (excluding the ovary) in the body. In this connection Lillie's remark that the foetuses must be protected in some way from the sex-hormones of the mother seems to have a possible significance.

"It is well known that the ovarian stimulus necessary for the original development of the reproductive organs is also necessary during adult life for the maintenance of those organs, and is therefore present during adult life. It is also known that this stimulus is hormonic in nature. Further, Lillie has shown that the subjection of a female foetus to the hormonic influence of a male foetus results in intersexual development, leading, in the case of the cow, to the animal known as the 'free-martin.' Considering these facts it is not unreasonable to suppose that, if the male foetuses in the normal pregnant animal were not by some means protected from the sex hormones of the mother, aberrations of sexual differentiation would occur. It is reasonable to infer, therefore, that some mechanism exists whereby the sex hormones of the mother are prevented from reaching the foetuses. In our view it is improbable that oestrin is the actual hormone associated with the original development of the accessory sexual organs of the female, but at the same time the above argument suggests very strongly that the concentration of oestrin in the placenta, where we are assuming it is not elaborated, might be the result of some protective retention on the part of the placenta. In other words, it seems possible that the placenta might withdraw oestrin from the circulation in order that the foetuses might thereby be protected." (Parkes and Bellerby [242].)

This hypothesis is, of course, incompatible with the work of Courrier [69] who reports that the hormone when injected into the guinea-pig during pregnancy passes across the placenta and affects the uteri of the female foetuses. Allen, Francis and Craig [18] have, however, failed to confirm this report on the rat and mouse.

16-2

Action in non-mammals. The action of oestrin in animals other than mammals does not appear to have been investigated to any extent. Riddle and Tonge [253] and Loewe, Voss and Paas [205] have to some extent investigated its action in birds.

V. THE REGULATION OF OESTROUS PERIODICITY.

(a) RÔLE OF THE CYCLIC STRUCTURES OF THE OVARY.

The intimate association between the ovarian and uterine cycles naturally led to the conclusion that the periodic growth of the cyclic structures of the ovary was responsible for the cyclic changes in the accessory organs. One view was that the corpora lutea of ovulation were the main regulators of oestrous periodicity, and that the corpora lutea developing after each ovulation inhibited the further development of oestrus during their functional lifetime. This view was supported by various experiments already mentioned, in which the excision of the corpora lutea of ovulation expedited the appearance of the next oestrus. In certain conditions, however, the absence of oestrus (as during anoestrus) is associated with ovaries containing no active corpora lutea, and consequently the next oestrus is produced without the preliminary atrophy of corpora lutea. For this and other reasons it is clear that the corpora lutea of ovulation cannot be the essential regulators of the basic periodicity of oestrus. If any cyclic structure of the ovary is responsible therefore for the regulation of oestrous periodicity it must be the maturing follicle. This conclusion is supported by a variety of observations.

(a) Oestrus first appears when the first ovulation occurs at puberty; during the whole of reproductive life follicular maturation is coincident with oestrus, and the last oestrus is synchronised with the last ovulation at the menopause.

(b) Where the breeding season is restricted, the natural beginning and end of the ovarian cycle coincides with the beginning and end of the cyclic uterine activity.

(c) In certain animals (ferret and rabbit) where ovulation is not spontaneous, the mature follicles and the oestrous condition persist together definitely in the absence of coitus.

(d) During times such as pregnancy, when no follicles normally mature, no symptoms of oestrus occur.

(e) A nymphomaniac condition of persistent oestrus is often found in conjunction with a cystic condition of the large ovarian follicles.

Relation between the Graafian follicle and oestrin. The general tendency to emphasise the importance of the Graafian follicle in the production of oestrus was accentuated when the liquor folliculi was found to contain large amounts of the oestrus-producing substance, and much of the extraction work has been carried out with liquor folliculi. Allen [6] claims to have shown that the amount of the oestrus-producing substance which can be obtained from the liquor folliculi varies according to the stage of development of the Graafian follicle, and increases during maturation. With regard to the oestrus-producing substance Allen [4] remarks: "Its presence and absence due to the periodic development of successive sets of follicles is sufficient to explain the mechanism of oestrous phenomena." This author

and his co-workers (10) actually maintain that the Graafian follicle elaborates the oestrus-producing hormone under the influence of the ovum itself. Hartman (135) and Robinson (254) also consider that the Graafian follicle both in its periodic appearance and in its hormone function is responsible for the periodic occurrence of oestrus. Loeb (182) also tends towards this view. Zondek and Aschheim (296) suppose the hormone to be elaborated by the *theca interna* of the follicle.

This hypothesis, however, has largely resulted from the elimination of other probabilities and not from an experimental basis. The only experimental work which appears to have been carried out directly to test the supposition has given conflicting results. Marshall and Runciman (218) failed to inhibit the onset of oestrus by rupturing the maturing Graafian follicles in the dog, and hence considered that the presence of mature follicles was not essential for the production of oestrus. Later, however, Marshall and Wood (219) were unable to confirm these results. Such experiments are, however, of a problematical nature, because (a) the operation of laparotomy and follicle puncturing is somewhat severe and might be followed by inhibitory effects of a purely post-operative nature, and (b) the ruptured follicles might produce luteal tissue with an inhibitory action.

During recent years, however, evidence has begun to accumulate that the periodic maturation of Graafian follicles is not the causative factor in the appearance of oestrus. Many years ago Blair Bell (35) reported experiments where the grafting of rabbit ovaries from which the cortical areas had been removed resulted in the appearance of oestrus in the host. Since the removal of the cortical areas of the graft would eliminate the vast majority, if not all, of the ovarian follicles, these experiments of Blair Bell's provided a hint that the Graafian follicles were not essential to oestrus production. The really crucial experiment, however, *i.e.* the investigation of the effects on the oestrous cycle of the entire obliteration of the follicular system of the ovary has only recently been attempted. Exposure to X-rays has long been known to cause degeneration of the Graafian follicles and the subsequent elimination of both the follicular and the luteal portions of the ovary. These effects have been investigated histologically, and to some extent physiologically, for many years (37, 38, 39, 43, 128, 276), and it has been shown that the obliteration of the follicles had no effect in inhibiting the development of the accessory organs of reproduction in the pre-pubertal animal, or in causing atrophy of the accessory organs in the irradiated adult. The early workers on irradiated ovaries, however, appear to have made no observations on the effect of X-ray sterilisation on the occurrence of the oestrous cycle. Recently, however, this problem has been investigated in some detail (Parkes and collaborators (44–47, 234–237)) and as a result of these experiments it is possible to say quite definitely that the entire elimination of the whole follicular system of the ovary does not bring about a failure to produce oestrus or any marked aberration in its periodicity.

Histological effects of exposure to X-rays. Early work on the histological effects of X-irradiation of the ovary was carried out by Halberstadter (129) on the rabbit. Bergonie, Tribondeau and Recamier (39) showed that the corpora lutea were found not to be affected, while Bouin, Ancel and Villemin (43) found that the interstitial

tissue remains intact. These authors have been confirmed in their main conclusions by many other workers. In the mouse irradiated before puberty (Brambell and Parkes (44-47)) the obliteration of the young Graafian follicles is followed by proliferation from the germinal epithelium, and, as the degenerating follicles are reabsorbed, this proliferation from the epithelium comes to form the whole tissue of the irradiated ovary. In certain abnormal instances this proliferation becomes of a luteal-like nature and as such may inhibit the occurrence of oestrus. Such abnormal cases are more common in animals irradiated at or before birth than in animals irradiated at weaning time. In the irradiated adult the histological changes are somewhat different. The corpora lutea remain intact and their actual reabsorption is delayed indefinitely, so that they become semi-permanent fixtures in the irradiated adult ovary. The reabsorption of the follicles in the adult does not proceed to the same extent as in the mouse irradiated before puberty, and the major portion of the irradiated adult ovary consists of diffuse and nebulous tissue which has originated from the disintegrating follicles. This development of epithelial tissue in the irradiated adult ovary is correlated with the absence of any proliferations from the germinal epithelium. In spite, however, of the follicular derivation of much of the tissue of the sterilised adult ovary, no signs whatever of any periodic change have been observed in the irradiated adult ovary once a stable condition had been arrived at.

The occurrence of oestrus after follicular ablation. In mice in which the entire follicular system has been destroyed by exposure to X-rays, the oestrous changes in the accessory organs occur as in the normal animal (Parkes (234-237)). The sterilisation of mice has been described at three times, (*a*) just before or at the time of birth, (*b*) at weaning time and (*c*) at maturity. In mice irradiated at weaning time the first oestrus occurs at eight to nine weeks of age as in the normal animal, and its subsequent periodicity, though slightly more erratic than in the case of the untreated animal, is reasonably normal. The cycle in animals sterilised before birth, or just after birth, tends to be more abnormal. This is apparently due to the fact that in certain of these early irradiated animals the typical post-irradiation changes are not found and the irradiated ovary comes to consist of tissue of a luteal type. In this latter group of animals the cycle does not occur, or else ceases after a transitory appearance. In the mouse sterilised while adult the oestrous cycle persists quite unchanged in the vast majority of cases. In 20 mice 116 cycles were observed before irradiation took place. The mean length of these was $5 \cdot 98 \pm 0 \cdot 153$. After irradiation 146 cycles having a mean length of $6 \cdot 64 \pm 0 \cdot 234$ were observed. This difference in length is barely of statistical significance and is clearly of no biological importance. The component parts of the cycle in the sterilised animal also showed this typical normality. The variability of the post-irradiation cycle was, however, found to be rather greater than that of the pre-irradiation cycles. In spite, however, of these minor deviations from the normal animal, it is quite evident that in the majority of cases the entire destruction of the Graafian follicles has no effect on the periodicity of oestrus.

Allen in a recent paper (8) has doubted whether any real information as to the course of events in the normal animal can be gathered from the occurrence of the

oestrous cycle in the sterilised animal. This objection might have weight if the sterilised animal were a separate and distinct entity from the normal one. In the animals sterilised when adult, however, the normal animal after irradiation, merges into the sterilised one by slow degrees. Even after perfectly adequate exposure to X-rays, follicular degeneration is comparatively slow and the irradiated animal, while becoming sterile, shows no abnormality of cycle. Since it is improbable that the periodicity of oestrus production could be taken over imperceptibly by a different mechanism during the process of sterilisation, it seems probable that the same mechanism of periodicity is at work in the sterilised animal as in the normal.

The fact that the obliteration of the cyclic structures of the ovary has no inhibitory effect upon the periodicity of oestrus production in the mouse, shows primarily three things: (a) that the Graafian follicle is probably not the essential source of the oestrus-producing hormone, (b) that the periodicity of oestrus is not regulated by the periodic maturation of follicles, (c) that since the elimination of the corpora lutea of ovulation has also no effect on ovarian periodicity, the corpus luteum of ovulation in the unmated mouse can apparently perform no inhibitory function such as has been demonstrated in the case of the cow (Hammond [132]) and the guinea-pig (Loeb [182]). It would seem probable that in such animals as the cow and the guinea-pig, in which the corpora lutea of ovulation have been shown to have an oestrus-inhibiting effect, the destruction of the cyclic structures of the ovary by exposure to X-rays would, by eliminating the corpora lutea, shorten the dioestrous interval. The same thing would be expected in the mouse after sterile copulation. Since the postponement of the next oestrous period by sterile copulation is almost certainly due to the stimulation of luteal tissue, the elimination of such tissue should put an end to the inhibition. Experimental work, however (Parkes: as yet unpublished), has shown that this is not actually found in practice. In certain cases the sterilised mouse, when mated, does actually copulate every four or five days, namely, at the periodicity characteristic of the unmated mouse. In the majority of cases, however, this is not found, and one or two oestrous periods, attended by copulation, results in a gradual fading out of the oestrous cycle. The possible meaning of this will be discussed elsewhere.

The non-essential nature of the Graafian follicle in the production of oestrin has been emphasised by quantitative examination of the liquor folliculi, and the residual tissue of the ovary after the removal of all large follicles (Dickens, Dodds and Wright [78], Parkes and Bellerby [239]). This examination has shown that the oestrus-producing hormone is fairly equally divided between the follicles and the stroma, and if the occurrence of the hormone in a particular tissue can be held to be evidence that it is produced there, it is clear that the stroma tissue of the ovary has at least as good a claim to be considered a site of origin as has the follicular tissue.

This X-ray work reveals the ovary in quite a new light, and two major problems are raised:

(a) If follicular maturation does not initiate oestrus, how is the synchronisation arranged?

(b) How is the periodicity of oestrus brought about?

The synchronisation of oestrus and follicular maturation. Instead of follicular maturation producing the oestrous stimulus, it seems probable that the correlation is exactly the reverse, *i.e.* that the oestrous stimulus produces follicular maturation. as well as the symptoms of oestrus in the accessory organs. This possibility has been suggested by recent experiments on the time relation between growth of the follicle and the beginning of operation of the oestrus-producing stimulus. It has been known for some time (Long and Evans (208)) that double ovariotomy may be followed shortly afterwards by the appearance of oestrus, although no subsequent occurrence is ever found unless regeneration takes place. The significance of this observation was, however, not early appreciated. In the mouse (Coward and Burn (74), Brambell and Parkes (48)) oestrus may appear up to 36–48 hours after double ovariotomy, and this can only mean that the oestrous stimulus becomes operative 36–48 hours before its effect can be detected by examination of the vaginal smear. Histological examination (Brambell and Parkes (48)) of the ovaries removed from animals which came into oestrus after the operation revealed the remarkable fact that the real maturation growth of the follicle only takes place after the oestrous stimulus has become operative. In the mouse the average volume of Graafian follicles which are not destined to ovulate at the next oestrous period is about 3,000,000 c.μ. This same size is maintained until half-way through the dioestrus after which they will ovulate. By the time that the oestrus-producing stimulus has become operative the follicles which will ovulate at that period have increased in size to about 3,500,000 c.μ. During the two days before ovulation takes place the increase in size of the follicle is enormous. When ovulation takes place their average size is between 8,000,000 and 9,000,000 c.μ. This can only mean that the real maturation growth of the follicle is not an inciting cause of oestrus production, and probably demonstrates that the maturation of the Graafian follicle is itself actually brought about by the operation of the oestrus-producing stimulus. One final proof is clearly needed to demonstrate this view, namely, it is necessary to show that the injection of oestrin will bring about follicular maturation when it would not otherwise occur. A number of experiments into which this point entered indirectly have been performed, but the conclusions which can be drawn from them seem to be conflicting. Frank, Kingery and Gustavson (119) claim to have caused ovulation in the immature rat by oestrin injection, but this is contrary to the results of Brouha and Simonnet (50) and Allen and Doisy (14) who produced oestrus but not ovulation. Parkes and Bellerby (241) failed to produce ovulation during lactation in the mouse by the injection of oestrin in quantities sufficient to bring on the ordinary symptoms of oestrus. Similar results were obtained by Slonaker (265) on senile rats.

The nature of ovarian periodicity. Since the cyclic structures of the ovary can no longer be held to be responsible, in the mouse at any rate, for the periodic occurrence of oestrus, it is necessary to form some working hypothesis to explain how non-periodic tissue can produce cyclic hormone effects. If it is assumed that the ovary, even in its non-cycle form, is the regulator of its own periodicity, it seems possible to explain this periodicity on either of two grounds.

(*a*) The hormone in question might be produced at periodic intervals and so achieve its periodic action. Against this possibility it must be urged that oestrin can be extracted from the ovary even when oestrus is entirely in abeyance, as during anoestrum and pregnancy.

(*b*) If oestrin is produced continually by the ovary as would appear to be the case from quantitative extraction work, it would seem that the periodicity must be regulated by some such mechanism as the periodic attainment of a threshold value. Such a hypothesis is, however, purely speculative and is difficult to apply to the normal animal.

The results obtained on the X-rayed animal were originally discussed on the lines indicated above in the supposition that the ovary regulation was internal. Recently, however, evidence has been accumulating rapidly that extra-ovarian influences at least play a certain part in the regulation of the ovary, and probably play a decisive one. This evidence is considered in the next sub-section.

(*b*) REASONS FOR SUPPOSING OVARIAN REGULATION TO BE EXTERNAL.

Up till quite recently the case for the hypothesis that the periodicity of the ovary depended upon its cyclic structures seemed so strong that comparatively little attention was paid to the facts which seemed to show that the regulation of ovarian periodicity is external to the ovary itself, and is therefore of somatic origin. The demonstration, however, that the essential basic cycle of the ovary is maintained even in the absence of the cyclic structures brought this problem to the fore. The grave difficulty of explaining how the basic ovarian cycle of periodic oestrus is maintained in such an animal as the sterilised mouse gives rise to suspicions that other factors than the ovary may be concerned in its regulation. Definite experiments pointing in this direction have been performed for many years.

Ovarian grafts. As long ago as 1900 it was shown by Foa[108] that very striking results were obtained by grafting ovaries from one animal into animals of a different age. Thus, if the ovary of an immature animal is grafted into an ovariotomised adult, the immature graft rapidly matures and reaches a state of maturity long before it would have done so in its original environment. Similar experiments have entirely confirmed these original observations and have added further details[172, 208]. Converse experiments of grafting an adult ovary into an ovariotomised immature animal is followed by no observable endocrine effects which are characteristic of the mature ovary, and the mature graft lapses into a state of disfunction[171]. Various control experiments have shown quite adequately that the effect on the graft is not due to any operative cause, and have proved that the ovarian age is regulated by the somatic age. Ovarian grafts into the male show equally striking results. Immature ovaries grafted into mature males cause a very rapid hypertrophy of the mammary apparatus, and the immature ovary, therefore, is equally capable of undergoing a rapid maturation in the adult male as in the adult female. Hammond[131] sums up these experiments as showing that "the age of puberty is determined by the nutritive state of the soma of the animal and not by age changes in the ovary itself." Ovarian grafts into the male, however, differ in some degree

from the grafts into the female. The graft into the female undergoes its normal cycle changes and ovulates, if possible, and produces corpora lutea. The graft into the male, on the other hand, fails to show its normal cycle and lapses into a state which in its endocrine effects, at any rate, appears to be analogous to a condition of perpetual oestrus (172). Most workers are agreed that corpora lutea are not formed in the ovarian graft into the male (33, 168, 256). These experiments suggest most strongly that the factor necessary for gonad maturation is present in both sexes in the adult animal, but they also suggest that the factor in the male does not produce the periodicity which is characteristic of the female. (See Lipschütz (171), for full discussion.)

Compensatory hypertrophy. A further group of facts which seems to show that ovarian regulation is at least partly somatic, relates to the compensating hypertrophy of the remaining ovarian tissue after the removal of half or more of the original ovarian tissue. In such cases the remaining tissue undergoes rapid hypertrophy and soon comes to function, as far as the production of follicles is concerned, to as great an extent as did the two complete ovaries. This fact has been expressed as the "law of follicular constancy" (171) and shows quite definitely that some factor in the body limits the number of follicles which can be matured in any given species of animals, and furthermore shows that this factor is somatic rather than ovarian. These facts have for long invited explanation, and it has usually been considered that some sort of metabolic mechanism is at work.

(c) THE ANTERIOR PITUITARY BODY.

Hammond (131) has put forward a view that certain substances essential for both growth and reproduction are present in the body in limited quantities, and that while growth is in progress, namely, during the pre-pubertal period, the reproductive function is of necessity in abeyance. When, however, growth ceases, the substances are released for the use of the reproductive system, and puberty supervenes. An extension of this hypothesis supplies some explanation of the absence of ovarian activity during pregnancy, because the substances in question to which Hammond has given the name "generative ferment" are in use for uterine and foetal growth. This hypothesis, of the somatic control of the ovary, though purely speculative, appears to have been partly vindicated by the recent work on the connection between the gonads and the anterior pituitary body.

It has long been known that the gonads are but one link in the general chain of internal secretions which is found in the body, and the exact nature of the relationship between the ovary and the other ductless glands has been a subject of much speculation. The sex changes which are known to follow the occurrence of cortical tumours of the suprarenal are well recognised, and the connection between the reproductive phases and the state of the thyroid and thymus glands have been known from antiquity (35). In the same way disorders of the anterior pituitary produce various aberrations of the reproductive cycle (35), and recently very striking work has been done on the relation between the ovarian cycle and the anterior pituitary body.

Sodium hydroxide extracts of anterior pituitary. The relation of the anterior pituitary to growth has been studied for many years, and in 1922 Evans (94), working on this aspect of the subject, noticed a remarkable effect produced in the ovary as the result of the injection of ox anterior pituitary preparations made by extracting with N/10 NaOH, neutralising and centrifuging. This effect consisted in the lutealisation of the ovary. All the follicles were caused to develop rapidly into luteal tissue, probably without the intermediate phase of ovulation. The luteal tissue produced by the initial injections could be caused to hypertrophy enormously as a result of further administration and ovaries could be produced consisting of many dozens of corpora lutea. In correlation with this development of luteal tissue, the changes characteristic of the oestrous cycle came to a complete stop, and this inhibition of oestrus was found to last for some considerable period after the termination of treatment. It was also shown that the injection of this anterior pituitary matter during pregnancy brought about a stimulation of the corpora lutea of pregnancy and resulted in their failure to atrophy at the proper time. As a result of this the period of gestation was unduly prolonged (Teel (280)). It was further shown that this luteal tissue would perform at least one other function of the corpus luteum, namely, the sensitisation of the uterus. Whereas placentomata cannot be produced in the ordinary unmated cycle in the rat, Long and Evans (208), and Teel (279) found that they could be caused to grow with regularity and vigour in the unmated mouse when injected with anterior pituitary extracts. Evans and his co-workers injected the extracts intra-peritoneally, and it has been suggested that the mere administration of any irritating material in such a way might easily cause aberration of the ovarian cycle. This criticism, however, can carry but little weight. It has been demonstrated that the same effects are brought about when the anterior pituitary extracts are administered subcutaneously (Parkes and Marrian—unpublished). When injected into the immature animal, the extracts, made as those used by Teel, caused the same sort of effect in making the ovary develop large amounts of luteal tissue. Neither in the immature nor the adult animal is oestrus a preliminary effect of injection of the anterior pituitary extract, and the production of luteal tissue appears to take place without ovulation or any other oestrous symptom. These anterior pituitary extracts have the same lutealising effect on the ovary of the sterilised mouse (Parkes and Marrian—unpublished).

Anterior pituitary transplants. During the last two years a remarkable extension of this work on the anterior pituitary has been made by Zondek and Aschheim (300-301) and Smith and Engle (88-91, 268-272). These workers have shown that most marked results can be obtained on the ovary by the transplantation subcutaneously of fresh tissue of the anterior pituitary. This transplantation, of course, consists of the injection of a macerated aqueous suspension and is only really a transplant in so far as certain tumours are transplanted in this manner. No graft is effected; the tissue is absorbed. The first effect of such transplantation in the adult is that all the Graafian follicles become stimulated and undergo rapid growth. Following this a super-ovulation occurs during which many dozens of ova may be ovulated at the same time. From these very numerous ruptured follicles a large number of corpora

lutea develop, and continued injection during this time results in the suppression of the noticeable external symptoms of oestrus, though mating will take place. If copulation takes place at one of these periods of super-ovulation, large numbers of embryos may be present in the uterus and a large proportion of these may become embedded and super-pregnancy may succeed for at least a short time.

In the young animal the effects of the transplantation of anterior pituitary is even more striking. The first effect is the appearance in about three days of a premature oestrus which is accompanied by all the normal oestrous symptoms, including the opening and cornification of the vagina, and also apparently by ovulation. This precocious oestrus may be induced as early as the second week in the life of the young mouse, namely, about the same time as the eyes open (272). Zondek and Aschheim (302) claim to have produced an aqueous extract with the same properties as the tissue suspension. The exact explanation of the discrepancy between the effects brought about by the injection of the sodium hydroxide extracts is not at the moment apparent.

Three vital differences exist between the two techniques:

(a) Absence of chemical treatment of Smith and Engle's preparations.

(b) Staleness of ox pituitaries compared with the freshness of the mice, rats, etc., glands used by Smith and Engle.

(c) Large amounts given by Evans and Teel. These authors injected daily amounts of extract corresponding to about 1 gm. of original ox tissue as compared with the two to three mouse pituitaries (? 3 mgms.) sufficient to bring on the result observed by Smith and Engle.

Smith and Engle's results would appear to explain the effect described in Section v (b) of transplanting the immature ovary into an adult environment, if it be assumed that only the adult pituitary has a stimulating action on the ovary. As a matter of fact, however, Smith and Engle (272) have shown that the immature pituitary will produce the same effects. This, of course, may be merely a quantitative effect of adding more pituitary to the immature animal. Engle (91) also extends their results to the interpretation of the facts of compensatory hypertrophy of ovarian fragments. This author found that a compensatory hypertrophy took place much more rapidly when assisted by the injection of anterior pituitary material, and conclude that the factor governing the constancy of the follicular number in any given species is located in the anterior pituitary.

The pituitary-ovary mechanism. Whatever may be the precise explanation of the discrepancy between the effect of the sodium hydroxide extract and the effect of the subcutaneous transplant, it is quite clear that the results make it necessary to consider very seriously the rôle of the anterior pituitary in the regulation of the ovarian cycle in the normal animal. In the light of these experiments it is necessary to suppose that the anterior pituitary body is a very considerable factor, if not the sole factor, in the regulation of the basic ovarian periodicity. Even if this generalisation is assumed, the precise nature of the periodicity is still not elucidated. By transferring the site of the periodic mechanism from the ovary itself to the pituitary, the study of the problem has really taken a step without having reached a solution.

The anterior pituitary appears to be devoid of cyclic structures, and it seems easiest to suppose, therefore, that the periodicity of the ovary is regulated by a periodic production by the anterior pituitary of the stimulating substance. The relation of the anterior pituitary mechanism to the causation of the anoestrous period is also discussed fully by Smith and Engle (272). Two points should finally be emphasised. Firstly, the whole of the effects of these anterior pituitary preparations on the accessory organs are brought about through the intermediary activity of the ovary. Neither of the anterior pituitary preparations show the least activity in producing effects in the ovariotomised animal, and there can be no doubt that the effects produced on the accessory organs are brought about by the induction in the ovary of the phase normally associated with them. The early induction of oestrous symptoms in the immature mouse by pituitary transplants is, for instance, identical with that brought about by the injection of the oestrus-producing hormone, with the exception that the latent period is a day longer when anterior pituitary transplants are made. This seems to show quite definitely that the oestrin production of the immature ovary has to be stimulated in order to produce these oestrous symptoms, and the extra day which is necessary for the effects of anterior pituitary transplantation to appear is probably occupied by this process. Secondly, if the basic cycle of the ovary is regulated by the anterior pituitary body, it is necessary to assume that the reproductive organs can also react upon the anterior pituitary. Thus, when the corpus luteum becomes persistent during pregnancy or pseudo-pregnancy, and oestrus is in abeyance, some means must exist whereby the periodic mechanism of the anterior pituitary can be geared to the ovarian and uterine cycles. The event of pregnancy, for instance, may act directly on the anterior pituitary and through this on the ovary, or the corpus luteum may be directly caused to become persistent and in turn throughout the cyclic mechanism of the anterior pituitary. No work on this aspect has yet been done: at present the question has not advanced beyond the stage of being a logical necessity.

VI. THE CORPUS LUTEUM.

(a) INTRODUCTION.

The basic ovarian cycle. The various lines of work which have been indicated above suggest fairly definitely that the mammal possesses a basic periodicity of oestrous production. This basic periodicity, which is probably regulated from extra-ovarian sources, is independent of the periodic growth of the corpus luteum. The cycle in the unmated mouse and rat consists purely of this basic periodicity and is unaltered by the obliteration of either follicles or corpora lutea. In most species, however, even in the unmated animal, this cycle is thrown out of gear by the transient development of corpora lutea after each ovulation, *i.e.* by the introduction of a luteal phase. Where the corpora lutea are caused to become fully developed by the incidence of pregnancy the derangement of the cycle is much greater.

The functions of the corpus luteum. The elucidation of the hormonic control of the luteal phase of the sexual cycle has not yet proceeded as far as in the case of

the follicular phase. Nevertheless, a great deal of work has been done on the functions of the corpus luteum, and, in spite of a present tendency to minimise the part played by the luteal tissue in the reproductive cycle, it is possible to attach to it certain definite functions. The history of the corpus luteum after its first formation following ovulation is dependent firstly on the species of the animal in question, and secondly, upon the actual occurrences taking place in the accessory organs. In the short five-day cycle of the unmated rat and mouse, the corpus luteum apparently plays no part and performs no function. After sterile copulation, however, the corpora lutea (of pseudo-pregnancy) undergo a greater development, and the postponement of the next oestrous period for a period of 12 days is correlated with the appearance of other luteal functions during the pseudo-pregnant period. For instance, both the rat and mouse develop the sensitivity of the uterus which is characteristic of the normal cycle in the guinea-pig, and which has been shown by Loeb's [178, 179] work to be dependent upon the presence of a functional corpus luteum. The same is found during lactation in the rat and mouse. During pregnancy the corpora lutea attain their maximum development, and their full functional activity is reached. The fate of the corpus luteum of ovulation in these animals is therefore dependent (a) upon whether copulation has taken place, and (b) whether this copulation was fertile, namely, dependent upon whether embryos become implanted or not. In the guinea-pig the normal unmated cycle includes a noticeable luteal phase which corresponds to the pseudo-pregnant period in the rat and mouse. In the rabbit no corpora lutea are found until copulation has taken place (ovulation in this species is dependent upon copulation) and no corpora lutea of ovulation alone are therefore found in this species. The corpora lutea in the rabbit, even when the mating has been sterile, undergo a noticeable degree of development and the result is the production of a pseudo-pregnant period. During this pseudo-pregnant period the mammary glands and the other accessory organs develop in a manner which is comparable with that found during early pregnancy, from which it differs only in the absence of embryos. In the dog [214] a noticeable pseudo-pregnant period is also found, but ovulation is spontaneous at oestrus and copulation is not required for the production of pseudo-pregnancy. There exists, therefore, a reciprocal co-ordination between the accessory organs and the corpora lutea. In the rat and mouse the corpus luteum of ovulation needs the stimulation provided by sterile copulation before it develops to the functional stage, and further, requires the stimulation provided by the presence of embryos in the uterus before the full development associated with pregnancy is produced. Following parturition, the fact that lactation is taking place will cause the corpora lutea of the post-partum ovulation to become persistent for a period of about three weeks. Just what may be the nature of the stimulation exerted by these occurrences in the accessory organs is quite unknown. Having, however, been stimulated by one or other of these occurrences the corpus luteum then proceeds to fulfil the functions which are associated with it.

The very numerous experiments which have been carried out on the experimental ablation and stimulation of the corpora lutea have made it possible to postulate that four functions are performed by the corpus luteum when it is caused to become

persistent by pregnancy, or in certain animals by pseudo-pregnancy and lactation. These functions are:

(*a*) The inhibition of oestrus and ovulation.

(*b*) The sensitisation of the uterus.

(*c*) The maintenance of pregnancy.

(*d*) The development of the mammary glands.

Whether the functions can all be carried out by one type of secretory activity is doubtful, and since the balance of opinion is now beginning to favour the hypothesis that the corpus luteum is derived from both membrana granulosa and theca interna (for discussion see Parkes and Bellerby [244]) it is possible that at least two different types of secretion are produced.

(*b*) THE INHIBITION OF OESTRUS AND OVULATION.

The idea that the corpus luteum performs the function of suppressing ovulation appears to have been put forward in the first place by Beard [34] and by Prenant [247]. These authors arrived at the conclusion on the basis of the general functional correlation which is found between the development of the corpus luteum and the absence of oestrus. This, of course, does not apply to the period of anoestrus during which both follicular and luteal development are in abeyance. This, however, is a special condition, and as regards the ordinary ovarian cycle of the regular polyoestrous animal the persistence of the corpus luteum is invariably associated with the absence of oestrus. During recent years this hypothesis has been extended in a number of directions.

(*a*) The removal of the corpora lutea of ovulation in certain animals such as the cow and the guinea-pig [132, 182] expedites the appearance of the next oestrous period. In the mouse, however, the elimination of the corpora lutea of ovulation by X-ray sterilisation does not bring about an earlier appearance of the next oestrus. This appears to be due to the fact that in this animal the dioestrous interval is very short and practically no development of the corpora lutea takes place in the ordinary unmated cycle. In other words, the unmated mouse possesses no luteal phase in the cycle and the elimination of the corpora lutea cannot eliminate a luteal phase. After sterile copulation, however, when the cycle does possess a luteal phase, the elimination of the corpora lutea has the effect of eliminating this phase. In the cow and the guinea-pig the luteal phase is prominent, and the elimination of the corpora lutea is able to expedite the appearance of the next oestrus.

(*b*) The abnormal prolongation of the functional life of the corpus luteum, either by experimental or pathological means, is followed by prolonged disappearance of oestrus. Thus, in the guinea-pig (Loeb [189]) hysterectomy results in the prolongation of the life of the corpora lutea for an extraordinarily long period, and this prolongation is accompanied by a cessation of the oestrous cycle. In the cow (Hess [145], Williams [290]) the persistence of corpora lutea results in sterility owing to the suppression of ovulation, and the expulsion of such corpora lutea by manipulation usually brings about the return of oestrus. In the same way the human

menstrual cycle may cease when the corpora lutea persist abnormally, and the removal of these is followed by the return of the cycle (Ochsnier (227)). There is thus good reason for supposing that the corpus luteum, when caused to persist either during the ordinary luteal phase in the unmated cycle, or else during pregnancy, or lactation (in the mouse), has the function of suppressing oestrus and ovulation.

Mechanism of oestrus inhibition. The mechanism whereby this action is performed is not definitely known. It is clear that it cannot be merely a local mechanical effect in the ovary itself, as might be supposed from the fact that competition must take place in the ovary between the follicles and the corpora lutea. The fact that the presence of a corpus luteum in one ovary is sufficient to inhibit the oestrus-producing activity of both ovaries, clearly invalidates this hypothesis. Thus, in the cow, and other normally monotocous animals, one corpus luteum only at one time is normally present. The same thing has been experimentally produced in mice by means of unilateral sterilisation.

By analogy with other ovarian functions it seems probable that the oestrus-inhibiting action of the persistent corpus luteum is brought about by means of some endocrine activity. Up to date, however, the suppression of oestrus in the normal animal by means of corpus luteum extracts has not been brought about with any great success. Corner and Hurni (65) report negative results with rats, while Loeb (184), working on the guinea-pig, was unable to produce regular positive results. Pearl and Surface (245) claim to have succeeded in stopping ovulation in laying hens by the injection of extracts of a commercial preparation of luteal tissue. Kennedy (152) reported positive results on the rabbit by the injection of saline extracts of dried (commercial) corpus luteum. Kennedy, however, says that ovulation was suppressed in some cases for a very prolonged period after the end of treatment, and in such a case the inhibition of ovulation can hardly have been comparable with the normal action of the corpus luteum. "Sterilising" effects of injections of corpus luteum extracts, which may be of an oestrus-inhibiting nature, have been described by Haberlandt (127, 128). Recently Papanicolaou (230) has described the suppression of oestrus in the guinea-pig by the injection of lipoid extracts of corpora lutea. No method of preparation is, however, given by this author. Papanicolaou attempted to assay his extracts by the time that oestrus was inhibited. Johnston and Gould (150) were unable to inhibit the action of oestrus-producing extracts by the simultaneous administration of luteal extracts made by the same method. The balance of evidence of this extraction work is therefore against the idea that the oestrus-inhibiting action of the corpus luteum can be reproduced by means of luteal extracts. It is obvious, however, that no amount of negative evidence from extraction work will show that no oestrus-inhibiting internal secretion is produced. The work so far carried out on the extraction of the corpus luteum may very well have failed to extract the hormone in an active form. Such a conclusion is suggested by the positive results obtained by Papanicolaou (230) and also those recently reported by the present writer and his collaborators (243). These workers have reported the extraction of active preparations of corpus luteum by the

following methods. The corpora lutea of the cow were dissected and all hollow specimens rejected. The solid corpus luteum tissue was then minced and ground up with anhydrous sodium sulphate. The mixture was extracted with ether extract, evaporated down to small bulk and precipitated with acetone. The acetone extract when evaporated down gave a brownish oil which, when injected (emulsified with $\frac{1}{2}$ per cent. sodium bicarbonate), was found to interrupt the oestrous cycle in the normal mouse. Large amounts of this oil had to be injected to produce positive results, but control experiments showed that the injection of even bigger amounts of inert fat emulsions had no effect on the cycle.

It may thus be said that indications are being obtained that the oestrus-inhibiting action of the corpus luteum may be simulated by the injection of corpus luteum extracts, and is, therefore, presumably endocrine in nature. In this case the interaction of the oestrus-producing hormone with the oestrus inhibitor offers a rich field for experimental work, and it seems probable that the easiest way to standardise the activity of oestrus-inhibiting extracts will be to assay the inhibitor against known amounts of the oestrus-producing substance. Preliminary experiments (Parkes and Bellerby [241]) on the interaction of the oestrus inhibitor and oestrus producer have been carried out by the injection of the normal animal during such times as pseudo-pregnancy and lactation when the corpus luteum is normally the dominating factor in the ovary and oestrus inhibition is found.

Injection of oestrin during lactation. During lactation the mouse suckling more than two young fails to show any signs of oestrus between the immediate post-partum oestrus and that which is found about 24 days after parturition when lactation is ending. This period therefore provides a convenient stage in the cycle for any attempt to override the oestrus-inhibiting action of the corpus luteum by the injection of oestrin. It was found (Parkes and Bellerby [241]) that the amount of oestrin required to produce oestrous symptoms during lactation was directly proportional to the number suckling. Since untreated mice will show oestrous symptoms if only two or less young are suckling, it may be said that under such conditions no oestrin is required to produce oestrous symptoms during lactation. When three young were suckling it was found that three or four M.U. were required to produce oestrus, while when the number suckling rose to seven, not less than 10 M.U. would produce oestrus. That this oestrus-inhibiting activity during lactation was actually due to the ovary, was shown by another series of experiments performed on ovariotomised lactating mice. After ovariotomy the inhibition set up by lactation disappeared almost entirely and bore no relation to the number suckling. It was clear, therefore, that the inhibition set up by lactation operates through the ovary and is not merely an effect of the heavy drain of lactation upon the metabolism. Further, it has recently been shown (results unpublished) that this oestrus inhibition found during lactation was not merely an ovarian effect, but was actually a luteal one. Unilaterally sterilised mice were allowed to become pregnant and to suckle their litter in the ordinary way. At the beginning of lactation the ovary containing the corpora lutea was removed and the sterilised ovary containing no corpora lutea left. In such animals the oestrus-inhibiting effect of lactation

is also negligible, so that the corpora lutea of lactation must be responsible for the oestrus inhibition.

These results imply an antagonistic action between the oestrus-producing hormone and the oestrus-inhibiting action of the persistent corpus luteum.

(c) THE SENSITISATION OF THE UTERUS.

Since the classic work of Fraenkel (109–111) it has been known that the presence of the corpus luteum is necessary for the attachment of the fertilised ovum to the uterine mucosa, and also for the subsequent maintenance of foetal nutrition. Fraenkel's work was put on a more satisfactory basis by the subsequent experiments of Ancel and Bouin (20–27) who, working on the rabbit, were able to extend the application of Fraenkel's results. After sterile copulation in the rabbit (which results in ovulation and the formation of corpora lutea) the uterus undergoes growth, vasculisation and glandular increase in a manner comparable to the growth changes during pregnancy. Similar changes occurring normally after oestrus (in the non-pregnant animal) were shown to occur by Hill and O'Donoghue (146) in *Dasyurus viverrinus*. These authors introduced the term "pseudo-pregnancy" for this condition. In the dog also, considerable uterine development is found after ovulation. The corpus luteum undergoes a marked degree of development and the uterine changes are correspondingly prominent. In the sow a certain amount of post-ovulation activity takes place in the uterus. There can be little doubt that this post-oestrous activity of the uterus represents the preparation of a suitable environment for the fixation of the fertilised ovum. In other mammals, however, the post-ovulation changes in the non-pregnant animal are much less intense, and the real demonstration of the connection between the development of the corpus luteum and the preparation of the uterus for the reception of the fertilised ovum has been made experimentally by showing that even in animals, where no great degree of post-ovulative uterine growth is found, the uterus is nevertheless in a peculiar condition, showing intense sensitivity to mechanical irritation. Thus Loeb (179), working on the guinea-pig, was able, by cutting the uterus, to cause the production of large blocks of tissue which resembled decidual cells and to which the name placentomata has been given. Loeb found that this sensitivity of the uterus after ovulation was entirely dependent upon the presence of corpora lutea and was produced at a certain period after ovulation. The removal of both ovaries, or the removal of the corpora lutea only, entirely inhibited this response of the uterus to mechanical irritation. In the case of the rat it was found by Long and Evans (208) that the uterus was quite unable to respond to such stimulation at any stage of the normal unmated cycle. These authors also found, however, that during pseudo-pregnancy in the rat, or during lactation (Corner and Warren (66), and Frank (113)), when the corpora lutea undergo a degree of development not found in the ordinary unmated cycle, this sensitivity of the uterus to mechanical irritation was found. These authors showed that the introduction into the lumen of the uterus of a small loop of surgical silk was sufficient to incite the growth of large quantities of decidua-like tissue. The uterus was found to be most sensitive

at about four days after ovulation. The fact that this sensitisation of the uterus does not take place in the rat during the ordinary unmated cycle is additional evidence that the corpus luteum of ovulation in the unmated cycle does not attain a degree of development required for the production of this sensitivity and is strongly reminiscent of the absence of effect on the periodicity of oestrus of the obliteration of the corpora lutea in the unmated mouse. It may be said, therefore, that in the rat and mouse the corpus luteum of the unmated animal possesses neither a sensitising nor an oestrus-inhibiting function. The work of Long and Evans [208] on the production of placentomata in the rat has been confirmed by the present writer on the mouse. The latter animal appears to be the same in all essential features, except that the time of maximum sensitivity is somewhat earlier after ovulation. Interesting experiments have recently been reported by Teel [279] which show pretty definitely that the corpus luteum in the rat is responsible for this sensitisation. In Section v (c) the experiments on the effect of sodium hydroxide extractions of anterior pituitary are recorded, and reference is made to the effect of this extract in stimulating luteal tissue. Teel found that the injection of the ordinary unmated rat resulted not only in the inhibition of oestrus, as has already been shown by Evans, but also in the occurrence of uterine sensitivity. The placentomata, which were produced most freely when the operation took place on the fifth day of injection, were not produced in the absence of ovaries, and were therefore due directly to ovarian activity and only indirectly to the anterior pituitary. There can be little doubt that this attainment of sensitivity was brought about by the hypertrophied luteal tissue of the ovary, whose growth had been stimulated by the pituitary extract.

The object of the post-oestrous activity of the uterine mucosa is clearly to facilitate the attachment of the fertilised ovum, but Loeb [180] found that implantation could take place, though rarely, in its absence. The nature of the activity by which luteal tissue sensitises the uterine mucosa is not definitely known, but Loeb [180] has reported two interesting facts:

(a) That the sensitisation is specific to the uterus. Other tissues are not affected.

(b) That the sensitisation is equally well performed in grafted uterine tissue.

It is thus fairly evident that the action is chemical, but it may not necessarily be of true hormonic nature.

Extracts of corpus luteum tissue capable of producing this sensitisation when it would not otherwise be found, do not yet appear to have been produced. Loeb [180] obtained only negative results by the injection into ovariotomised animals of corpus luteum extracts and also by the injection of blood from animals in the stage of sensitivity. These results are, however, merely inconclusive, and little doubt can be entertained that future work will result in the preparation of sensitising extracts. Whether or not this function is also performed by the oestrus-inhibiting extracts described in the previous section is not yet known.

(d) THE MAINTENANCE OF PREGNANCY: PARTURITION.

Removal of the corpus luteum during pregnancy. Considerable experimental work has failed to elucidate the exact degree to which the corpus luteum is necessary for the maintenance of pregnancy. As pointed out in the previous section, there is little doubt that the corpus luteum is necessary for the sensitisation of the uterus, and is thus almost essential for implantation. Authors are, however, not agreed as to how long the corpus luteum is subsequently required. Fraenkel [110], who failed to find that the corpus luteum was necessary during the whole of gestation, came to the conclusion that it was only required during the early stages after implantation. He found that its removal during the first half of pregnancy regularly led to abortion. Marshall and Jolly [215] for the dog and the rat, and Kleinhaus and Schenk [153] and Daels [76] for the rabbit, guinea-pig and the rat, came to the same conclusion. Clinical cases reported by Blair Bell [35], and Essen-Moller [93] suggest that in the human subject the removal of the corpus luteum of pregnancy during the later stages had no adverse results. In spite, however, of the large amount of evidence which seems to show that the corpus luteum is not essential for the whole of gestation many other authors have found that the removal of the corpora lutea at any stage results in the termination of pregnancy. Blair Bell and Hick [36], Hammond [131] and Weymeersch [289] have reported that the removal of the ovaries during pregnancy in the rabbit is inevitably followed by reabsorption or abortion. In the cow the removal of the corpora lutea was found by Hess [145], Wester [288], and Schmaltz [257] to be incompatible with the normal progression of pregnancy. Similar results have been described for the goat (Drummond, Robinson and Asdell [86]), the opossum (Hartman [137]) and the spermophile (Drips [85]). Further evidence may be drawn from the pathological cases where, for some reason or other, the corpora lutea have undergone degeneration during pregnancy. Hammond [131] and Hartman [138] have shown that foetal death normally follows such degeneration.

The difficulty of satisfactorily removing the luteal tissue without removing the entire ovarian activity by double ovariotomy has been got over in recent experiments on the mouse by means of unilateral sterilisation (Parkes [238]). In these experiments unilaterally sterilised mice were allowed to become adult, and eventually to become pregnant, from the one sound ovary. Pregnant mice were thus produced with one normal ovary and with one sterilised ovary, the sterilised ovary possessing no corpora lutea, but being capable, as was already known, of performing all ovarian functions other than those associated with the corpus luteum. In such mice the removal of the sound ovary containing the corpora lutea resulted invariably in the termination of pregnancy, while the removal of the sterilised ovary failed to produce any results. These experiments demonstrated that in the mouse, at any rate, the presence of the corpora lutea of pregnancy is necessary during the whole of gestation, and that in the absence of such corpora lutea abortion or reabsorption results.

There is very considerable discrepancy, therefore, between the results of various workers in this field, and at the moment it is impossible to draw any general conclusions as to the necessity or non-necessity for the presence of the corpora lutea

during pregnancy. The variability of the results may be to some extent due to the variation in the methods used to remove the corpora lutea and to the variety of animals used. It is improbable that in closely related species any great difference is to be found in the necessity for the presence of the corpora lutea of gestation, but it is quite probable that in a widely different species a difference in requirement may be found. The methods used to eliminate the corpora lutea clearly fall into two classes: (*a*) those which remove the corpora lutea only, and (*b*) those which result in the total elimination of all ovarian activity. The first class includes, of course, the cauterisation of the corpora lutea, the surgical dissection of the corpora lutea from the rest of the ovary at the time of operation, the squeezing out of the corpora lutea as performed in the cow, and the unilateral sterilisation technique. The second class includes double ovariotomy.

The causation of parturition. The distinction between double ovariotomy and the removal of the corpora lutea only is of importance, because it is possible that the abortion brought about by the removal of the corpora lutea is analogous with the parturition which sets in when the corpora lutea of pregnancy undergo their normal atrophy, and may be due to the premature operation of a parturition mechanism. Dixon and Marshall (80) have shown that the mechanism of parturition probably depends upon the stimulation of the posterior lobe of the pituitary by the immediate pre-partum ovary. These authors found that extracts of the ovary just before parturition (after the degeneration of the corpora lutea) when injected into the dog, caused an increase of pituitrin in the cerebro-spinal fluid as tested on the guinea-pig's uterus. They interpreted this result to mean that the ovary in the stage immediately before parturition and when freed from the dominating influence of the corpus luteum, produces some substance which has a stimulating effect on the posterior pituitary. On this view parturition is a positive action on the part of the ovary and the abortion which follows double ovariotomy during gestation cannot, therefore, depend upon such a mechanism. Double ovariotomy by removing all ovarian functions would make it impossible for a parturition mechanism depending upon positive ovarian action to be set in motion. The removal of the corpora lutea alone, on the other hand, would allow the ovarian stroma to perform this positive function. In other words, the abortion following removal of the corpora lutea can be explained on Dixon and Marshall's hypothesis of parturition only when the removal of the corpora lutea is brought about without eliminating other ovarian functions. Most authors who have found abortion to follow the removal of the corpora lutea have, however, performed the elimination by means of double ovariotomy, and there seems no doubt that in some of these cases the result is genuinely due to the removal of the corpora lutea and not to operative or other effect. It is necessary, therefore, to explain these results either by means compatible with Dixon and Marshall's theory of parturition by supposing that the experimental abortion has no relation to ordinary parturition, or else to explain the results in keeping with some other hypothesis of the conditions which bring about parturition. In this connection recent experimental work by Knaus (154) is of interest. This worker found that the susceptibility of uterine muscle varied

greatly during pregnancy, but found that the susceptibility was greatest at the very end of pregnancy. During the main period of gestation the sensitivity to pituitrin was very definitely subnormal. Knaus controlled his experiments very carefully, and eliminated any possible result of the increased length of muscle fibre which is found during pregnancy by using rabbits in which one cornu of the uterus had been rendered sterile by unilateral ovariotomy. The unstretched sterilised cornu was used for the actual isolated muscle experiments. Knaus explains his results on the grounds that the corpus luteum during pregnancy causes the sensitivity of the uterus to pituitrin to decrease and when the corpus luteum atrophies at the end of pregnancy the sensitivity returns to normal. On this view the corpus luteum is just as much the initiator of parturition as it is on the hypothesis put forward by Dixon and Marshall, but on Knaus's view the ovarian action in precipitating parturition is what may be called negative, and depends purely on the atrophy of the corpora lutea. Knaus's view, therefore, explains why the elimination of the corpora lutea alone, and also the ablation of all ovarian activity by double ovariotomy, should both have similar effects in bringing about abortion.

It is quite possible, of course, that the failure of the uterus to retain the foetuses after the removal of the corpora lutea has no relation to the normal parturition mechanism, and in this case the evidence from the experiments on the induction of abortion cannot be used to support or refute either the view of Dixon and Marshall [80] or the supposition put forward by Knaus [154]. In the circumstances, therefore, it is not possible to do more than say that if the induction of abortion by the removal of the corpora lutea has any relation to the normal parturition mechanism, then evidence against the view of Dixon and Marshall is obtained.

Prolongation of gestation by luteal stimulation. Since the atrophy of the corpora lutea is a necessary prelude to parturition it would seem that the injection of active luteal extracts when this atrophy is beginning should inhibit the onset of parturition. Experimental data on this point appear to be lacking (preliminary work with the oestrus-inhibiting extracts of corpora lutea has, however, given some inconclusive results).

Remarkable results have, however, been obtained by Teel with the injection of the NaOH extract of anterior pituitary during pregnancy. Injected at this time it produces its usual luteal stimulation, and apparently as a consequence of this, the period of gestation was lengthened from two to six days. Teel's conclusions are as follows:

(*a*) The increase in the gestation period is due to a delay in implantation of from three to six days.

(*b*) Eventually the foetuses die *in utero* (apparently owing to failure of the parturition mechanism), and are expelled stillborn.

(*c*) This intra-uterine death is due to severance of the placental attachment.

The delay in implantation of the fertilised ovum is not what would have been expected from the experiments reported in Section VI (*b*), but, even if the lutealisation of the ovary during pregnancy does not make possible the greater development of the foetus, it clearly interrupts the normal mechanism of parturition.

This clearly offers a rich field for future work, especially the comparison of the effects of the NaOH lutealising extract of anterior pituitary, with those produced by the aqueous suspension of the lobe. Zondek and Aschheim (302) report the induction of ovulation during pregnancy by means of the injection of the aqueous suspension, but the course of gestation was apparently not interrupted.

Relation between oestrus and parturition. It is necessary to mention here the experiments dealing with the induction of abortion by the injection of the oestrous hormone. Allen, Francis and Craig (18) obtained a positive oestrous smear during early pregnancy by injection of oestrin, but Brouha and Simonnet (49) failed to do so in the later stages. Smith (266) showed that pregnancy in the rat could be interrupted in its early stages by the injection of the oestrus-producing hormone. This author, however, failed to produce this result in the later stages. Parkes and Bellerby (240), however, showed that in the mouse pregnancy could be terminated at all stages by the administration of an adequate dose. The amount required during the later stages was, however, roughly twice as great as in the early stages. It is probable that this greater dosage in the later stages is made necessary by the greater development of the corpora lutea at this time. Future work may show that the amount of oestrin required to produce abortion is proportional to the number of foetuses *in utero* (or more correctly to the number of corpora lutea in the ovary), but the methods available so far have not been sufficiently accurate to demonstrate such a correlation. Loeb and Kountz's (192) failure to interrupt pregnancy in the guinea-pig was probably due to specific differences or to the use of inadequate doses. The facts as regards mice and rats seem to be well authenticated. The result is probably brought about by the physiological elimination of the corpora lutea consequent upon the overriding of their function by excessive quantities of the oestrus-producing hormone. If this is so, a superficial analogy with the operative removal of the corpora lutea is obtained. Further, it is possible to compare this induction of oestrus during pregnancy with the oestrus which is found immediately after parturition. In the rat and mouse, at any rate, oestrus and parturition are closely connected, and parturition may be almost regarded as a pro-oestrous symptom of the immediate post-oestrous period. It is possible, therefore, that the substance extracted from the pre-partum ovary by Dixon and Marshall (80), and injected into the dog was actually the oestrus-producing substance of the ovary. The fact that their extracts were made by simply boiling the ovaries with saline and filtering is, of course, against this, because the oestrus-producing hormone would hardly be extracted by this process. Also, experiments on dogs have failed to show that the injection of the oestrus-producing substance results in any hyperactivity of the posterior pituitary. On the whole, therefore, though the possibility still exists, the probability is against the idea that even in the rat and mouse parturition is initiated by the imminence of the next oestrous period. Many mammals, of course, show no connection between parturition and oestrus. Actually, therefore, the abortion brought about by injection of oestrin during pregnancy is probably simply due to the overriding of the corpora lutea and to the indirect stimulation of the parturition mechanism only.

(e) THE DEVELOPMENT OF THE MAMMARY GLANDS.

The work which has been discussed in Section IV makes it clear that two stages of development take place in the mammary gland, even in the animal before the first ovulation:

(a) A slight and gradual pre-pubertal development.

(b) A burst of growth at the first and following oestrous periods.

This growth still leaves the gland in a rudimentary condition, and the removal of the stimulus never results in the secretion of milk. (See below.)

The mammary gland in pseudo-pregnancy. During the presence of the persistent corpus luteum, however, an entirely new phase of growth is initiated, and the removal of the stimulus during the later stages of this growth results in at least a temporary secretion of milk.

In the rabbit, for instance, the original animal may stay in a condition of oestrus for some months, in the absence of copulation, but in spite of this continued activity of the oestrus-producing hormone no development of the mammary glands takes place other than the slight growth normally associated with oestrus. Immediately ovulation takes place and the corpora lutea are formed, however, growth starts and, even in the absence of pregnancy, continues for some 14 days. This proliferation consists in the lateral extension and swelling of the ducts. During pregnancy, the history of the mammary glands in the rabbit is the same as in pseudo-pregnancy for the first 14 days, but following this period, instead of the katabolic processes which occur after pseudo-pregnancy, the glands enter into a new phase of development which consists of thickening of the mammary tissue, as well as in further lateral extension. This phase proceeds till the end of pregnancy, when the breakdown stage sets in.

In the dog (Marshall (214)) development of the mammary glands is also found during pseudo-pregnancy, and in this animal the anabolic phase may proceed so far that the breakdown process at the end of pseudo-pregnancy may actually lead to lactation. A similar state of affairs is found in *Dasyurus* (Hill and O'Donoghue (140)).

In the guinea-pig the mammary tissue (Loeb and Hesselberg (190, 191)) undergoes a certain amount of development during the luteal phase of the ordinary cycle in the unmated animal, and this development is exaggerated when the corpora lutea are caused to become persistent by hysterectomy. During the ordinary dioestrous cycle, however, even where a luteal phase (as in the guinea-pig) is found, the development of the mammary glands is not normally pronounced enough to result in the actual secretion of milk. Hammond and Woodman (133), however, report that virgin heifers, after a series of dioestrous cycles, may occasionally secrete a small quantity of milk. None of the mammary changes characteristic of pseudo-pregnancy are found after ovariotomy or removal of the corpora lutea.

The control of mammary development is, of course, known to be endocrine in nature (grafting experiments, etc., see Marshall for full discussion), and the observations on the glands during pseudo-pregnancy make it evident:

(*a*) That the presence of foetuses is not essential for the development of the mammary glands as found during at least the early stages of pregnancy.

(*b*) That since the only ovarian change in pseudo-pregnancy is the development of corpora lutea, it is reasonable to suppose that these bodies are the activating factor. The almost synchronous appearance of the katabolic changes in both the corpus luteum and the mammary tissue further supports this view.

The induction of mammary growth to a degree comparable with that found during pseudo-pregnancy by the injection of corpus luteum extracts into the ovariotomised animal does not, however, appear to have yet been satisfactorily performed. Preliminary experiments with the oestrus-inhibiting extract of the corpus luteum have given negative results. Loeb [184] failed to cause mammary development by injection of aqueous extracts of corpus luteum. Champy and Gley [60] have recently reported the induction of mammary growth by the injection of luteal extracts, while Beucan, Champy and Keller [40] claim that the corpus luteum substance can be obtained from placentae as well as the follicular hormone. The present writer has, however, by the injection of the NaOH extract of anterior pituitary, induced in the non-mated and therefore non-ovulating rabbit a degree of mammary growth in excess of that found during pseudo-pregnancy. This result appears to be due to the lutealising effect of the pituitary extract upon the ovary (equally clear in the rabbit as in the rat and mouse: see Section v).

The cause of the final development during pregnancy. Although it is tolerably certain that the initial growth of the mammary gland during pregnancy is brought about by the corpus luteum, it is still far from certain whether the whole growth characteristic of pregnancy can be ascribed to the same source. In view of the alleged functionless state of the corpus luteum during the later stages of pregnancy, certain authors have supposed that some other factor must be sought, but since the evidence is at least as strong that the corpora lutea function all through pregnancy (see Section vi (*a*)), this argument has little force. The fact that during pseudo-pregnancy the development is not complete is also not valid, as the withdrawal of the luteal stimulus probably precedes the degeneration of the mammary gland at the end of pseudo-pregnancy.

On the other hand, the fact that complete lactation takes place in the egg-laying mammals suggests that no foetal factor is necessary. Hammond [130] also has shown that the presence of decidual tissue during pseudo-pregnancy does not increase the mammary development. These and many other observations are cited against the view that pregnancy is necessary to complete the normal growth of the gland found at that time. Much of the evidence often put forward is, however, irrelevant. It cannot be denied that in the absence of foetuses the mammary gland can be built up to a degree sufficient for the transient secretion of milk. The real question is whether the final development of the glands (so clearly seen in the rabbit) can be achieved in the absence of pregnancy. It is quite probable that some product of conception is necessary to complete the development.

Ancel and Bouin [24] postulate the presence of a "myometrial" gland during pregnancy, which is responsible for the later stages of development.

Lane-Claypon and Starling (156) produced development of the mammary glands in virgin rabbits by means of injection of extracts of embryos. The development produced, however, appears to have been that typical of early pseudo-pregnancy, rather than that found in late pregnancy.

It should be possible to answer the question finally by causing pseudo-pregnancy to last indefinitely by the injection of the luteal stimulating extracts of anterior pituitary.

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THE INFLUENCE OF ULTRA-VIOLET LIGHT ON PLANTS

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THE various effects of visible light on plants became the subject of much observation and experiment during the greater part of the nineteenth century, but the effects of ultra-violet rays have been explored only in recent years, and their possibilities are still comparatively little known. Investigations along these lines have been much stimulated by the rapid developments in our knowledge of the therapeutic effects of ultra-violet light and especially of the so-called "biologic" rays.

The limit of the visible spectrum in the violet region has rays of wave-length 4000 Å.U. and beyond this there are invisible radiations of progressively shorter wave-length, though of course with no sharp line of demarcation between the two. The whole series may be obtained by the use of suitable incandescent substances, but in sunlight and clear air the solar spectrum ends abruptly in the region of 2900 Å.U.[1], so that in nature vegetation may be exposed to invisible radiations of 2900–4000 Å.U. The amount of ultra-violet light reaching the earth's surface is very variable, depending on altitude, atmosphere and on the season of the year. Of the ultra-violet rays present in the solar spectrum under favourable conditions, it is those of shorter wave-length (λ 2900–3200) which have been found of great benefit to animal life, especially those of wave-length 2900–3200 Å.U. and these are the so-called "biologic rays": the longer λ 3200–4000 appear to have little effect. Evidence is accumulating which suggests that both may also be beneficial to plant life.

Within the last two years the attempt has been made to utilise the ultra-violet of sunlight in horticulture by the use in greenhouses of glass transparent to these rays, such as Corning glass in America or Vitaglass in England: it is too soon to judge of the results of these experiments.

Ultra-violet light from artificial sources includes wave-lengths ranging usually from 1600 to 4000 Å.U., varying with the source of light: rays of wave-length shorter than 2900 Å.U. have a lethal action on infusoria and it has been found that they may also have a harmful effect on plant life.

As early as 1904, Hertel examined bacteria and also leaves of *Elodea* under a microscope provided with a quartz condenser and illuminated with monochromatic light of wave-length 2800 Å.U. (the magnesium spectral line). Movement of the bacteria and protoplasmic streaming of the cells of the *Elodea* leaf were retarded

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[1] Å.U. = "Ångström" units; there are 100,000,000 Å.U. to the centimetre. Wave-lengths are also sometimes expressed in terms of μ or $\mu\mu$ and their relation is such that $1\mu = 1000\mu\mu = 10,000$ Å.U.

and finally inhibited altogether. In the presence of white light this effect was less marked. By means of a sensitiser, such as eosin, he could obtain a similar effect with yellow light, and he therefore considered that the injury was associated with the absorption of the short rays by the tissues, a conclusion which has been amply justified by subsequent results along other lines.

In 1910 Schulze tried the influence of monochromatic light of the same wavelength on the stripped epidermis of leaves of *Ficus*. He found that the epidermis was quite opaque to the light and concluded that the cuticle might afford protection against the ultra-violet rays of the sun, as had already been suggested by Köhler (1904); this cannot, however, be maintained, since it is now known that the ultra-violet of sunlight does not extend, at any rate in Europe or North America, to wavelengths shorter than 2900 Å.U. even under the most favourable conditions. Other plant products—woody walls and cork—had also been shown by Köhler to be opaque to the light from the cadmium line (2750 Å.U.).

More recently the polychromatic radiations from a quartz mercury vapour lamp or from a carbon arc have been used, the exposures of the plants often being severe. Spectral analyses show that in the former case the shortest rays are of about 2100 Å.U., and that in addition there are bright lines in the blue and green regions of the visible spectrum. This furnishes a steady, cold light for short intervals, but the intensity is apt to fall off considerably with the age of the lamp. The carbon arc has bright bands in the ultra-violet, a continuous spectrum of visible light, and also furnishes a considerable amount of heat and is a more steady source of light over long intervals. In both types the intensity of light depends on the amount of electrical energy consumed and it was shown by Angus (1925) that the lethal effect of a carbon arc lamp on infusoria also depended on the electrical energy consumed. Colourless cultures of *Paramoecium* were used, in which the organisms were only thinly distributed. Tests were made by varying the current passing through the arc, the voltage between the carbons and the length of the arc, but keeping the total consumption of energy constant.

In 1911 Kluyver obtained many interesting results, using the full radiation of a quartz mercury vapour lamp (Heraeus, 220 volts, $3\frac{1}{2}$ amps.), which was placed in a dark room in a casing of blackened wood. The plants were irradiated at a distance of 30–40 cm. in front of it for periods varying from a few minutes to several hours. In the latter case a rise of temperature at the surface of the plant of about 4° C. was noticed.

Leaves of *Aucuba japonica* were exposed at 40 cm. distance, the under surface being towards the source of light. After 20 minutes black paper was placed over one end of the leaf to screen it, whilst the exposure of the rest of the lamina was continued. At intervals the paper was advanced further, so that each new strip covered had progressively longer irradiation than the one preceding it, the last having received as much as $2\frac{1}{2}$ hours. No visible change was noticed at the end of the experiment, but next day darkening was seen in all the strips exposed for $1\frac{1}{3}$ hours or more, and after 3 days all those which had received over 40 minutes' irradiation had turned black (the usual post-mortem change in these leaves), whilst the remainder of the

leaves were still bright green. The leaves of numerous other plants (*Ficus, Hedera, Taxus, Corylus*, etc.) were examined and, although their resistance was varied, yet in all, if the initial exposure was not too great, a latent period elapsed after which signs of injury became apparent. The injury was usually confined to the epidermis, excepting in long exposures, as when young stems of *Phaseolus* were irradiated for 6 hours, when four layers of the underlying cells were killed. Young stems or leaves were usually more sensitive than older ones and in *Aucuba* the stomata were more resistant than any other epidermal cells. The latter conclusion was also reached by Ursprung in 1917 for other plants. Recent observations show that this is by no means always the case, but that in *Voandzeia subterranea*, a leguminous plant with mesophytic leaves, the reverse is true, whilst in *Trifolium subterraneum*, also a mesophyte, the stomata collapse first as in *Aucuba* (Delf, Ritson and Westbrook, 1927).

Kluyver also demonstrated that the epidermis of *Ficus elastica* when closely applied to the surface of the leaf of *Aucuba japonica* gave complete protection from subsequent irradiation with a quartz mercury vapour lamp. He also irradiated the leathery leaves of a plant of the high Alps, *Homogyne discolor*, and found that after 5 hours' exposure the leaves were killed. He concluded that alpine plants were not protected in nature from the shorter rays of the ultra-violet, if these were present in the solar spectrum.

In 1917 Ursprung and Blum investigated afresh some of the harmful effects of ultra-violet light on plants, using a small quartz mercury vapour lamp (2 amps., 37 volts) at a distance of 20 cm. from the experimental object. Temperature effects at the surface of the plant were avoided by means of screens of quartz glass. A variety of leaves was employed and the criterion selected in most cases was the absence of injury, after plasmolysis of a strip of the epidermis previously treated with a slightly hypertonic solution of cane sugar, uninjured cells giving complete deplasmolysis on irrigation again with water.

By this means it was found that in green leaves the epidermis was mainly concerned, the mesophyll being entirely protected: in variegated leaves of *Pelargonium* the white parts were more resistant than the green, whereas in *Vicia* the reverse was the case. In *Zea maïs* old leaves were found to be more resistant than young ones, unlike those of *Aucuba* described by Kluyver. In the parti-coloured flowers of *Viola tricolor* the epidermis of the yellow region was much more sensitive than that of either the blue or the white. In the red blotched leaves of *Coleus Blumei* all the cells were killed in the red region after an exposure of only half an hour and also about 80 per cent. of the mesophyll cells below. These and similar observations suggest that the presence of pigment leads to increased absorption of the ultra-violet rays, as would indeed be expected, but the results were not all harmonious and the subject would probably repay further investigation. The work of Bovie (1913) and of Dreyer and Hansen (1917), showing that proteins *in vitro* (globulins, albumin and fibrinogen) can be readily coagulated by irradiation with ultra-violet light, makes the injury to the protoplasts of superficial cells readily explicable.

Stoklasa in 1912 compared the behaviour of etiolated seedlings of *Pisum sativum*, *Zea maïs*, *Avena sativa* and *Hordeum distichum* when exposed to sunlight and to the

light of a quartz mercury vapour lamp (4 amps., 110 volts) at a distance of 45 cm.; in each case the chlorophyll formation appeared first in the irradiated seedlings. The same result was observed when the lamp was covered by a glass globe cutting off all the shorter ultra-violet rays, and Stoklasa concluded that the ultra-violet rays of greater wave-length (3000–4000 Å.U.) were the effective cause, but he took no account of the visible light from the blue and green regions in the spectrum of the mercury vapour lamp nor of any local temperature effect, although rise of temperature is known to accelerate the rate of production of chlorophyll. The same objections apply to the work of Russell and Russell (1925) and it therefore still remains to be seen whether ultra-violet light has any influence on the development of chlorophyll.

The general effects of single exposures to ultra-violet light from artificial sources may be summarised briefly as:

(1) Absorption of the rays by the epidermis and cuticle.

(2) Latent period without visible changes.

(3) Lethal effect in the epidermal cells if the exposure has been of sufficient duration.

The individual cells may react differently, as in the case of the stomata and adjoining cells, and the age of the leaf influences the result, but in a variable way in different leaves.

A somewhat different line of investigation involves the use of continual or repeated shorter exposures to ultra-violet light and observations of the subsequent and cumulative effects on growth. In 1913 Raybaud grew cress seedlings under continual illumination at a distance of $1\frac{1}{2}$ m. from a mercury vapour lamp. The seedlings germinated but were stunted and distorted, with a papery brown surface composed of the collapsed and shrivelled epidermal cells; these appeared to form a sufficient protection to the cells beneath.

Experiments by Russell and Russell (1925), using a Hewittic mercury vapour lamp were made on mustard seedlings, the exposures being given at a distance of 2 feet and varying from 5 to 50 minutes daily. In every case there was some stunting of growth and foliage and this was greater in the case of seedlings grown otherwise in darkness and less when given to plants grown otherwise in normal daylight. The experiments were not carried far enough to give indications as to the resultant effects on structure and reproduction. In the same year the results of an extensive series of experiments on the influence of ultra-violet light from a quartz mercury vapour lamp on seedlings grown otherwise in normal conditions was also briefly reported by Popp (in Ellis and Wells, 1925 p. 285). Details are not given in this preliminary account of the results obtained, but the observations on germination and subsequent growth of the seedlings indicated the following amongst other results:

(1) Exposures of more than 2 hours to the full light of a quartz mercury vapour lamp decreases the rate and amount of germination, wave-lengths of less than 3000 Å.U. being the most effective.

(2) In seedlings the younger leaves are the most susceptible to injury.

(3) Bacteria and other fungi causing damping-off do not develop under the full light of a mercury vapour lamp, but will do so if the rays below 3000 Å.U. are removed by screening.

In the years 1925–7 rapid advances were made in the development of new types of lamp, in methods of standardising and comparing the nature and intensity of their illumination, principally from the medical point of view, and also in the production of glass of special composition suitable for screening off certain rays. The absorption factor for such types of glass is known in a general way, but there are great practical difficulties in obtaining equal illumination owing to loss of light by absorption, to unequal distribution of energy in the different parts of the spectrum, and to variations in the transparency and also the thickness of different pieces of glass of the same kind. This difficulty has been partly overcome by the use of biological tests, such as the time required just to produce erythema in the human forearm at a specified distance, or the killing power in the case of certain infusoria. Chemical tests such as the acetone methylene blue (Webster, Hill and Eidenow, 1924) and lithopone reactions (Clark, 1924) have been correlated with these biologic ones but are mainly sensitive to wave-lengths of 2900–3200 Å.U.— the rays which are also most active in the production of erythema.

In 1927 Sheard and Higgins reopened the question of the effect of ultra-violet light on the growth of seedlings, of lettuce, radish, cucumber and turnip, giving them daily irradiations at a distance of 50 cm. from an air-cooled mercury vapour lamp, half being kept otherwise in darkness and half in diffuse daylight, under similar conditions as to temperature and moisture. These seedlings show normally much greater capacity for germination in darkness than in light. In the experiments they were irradiated daily for 1–60 minutes in lots:

(*a*) with the full light of the lamp;

(*b*) with the lamp screened by window glass, cutting off all the ultra-violet rays except those of 3200 Å.U. and longer;

(*c*) with a Vitaglass screen, cutting off all rays shorter than 2700 Å.U.;

(*d*) with a dark screen of "Corning Ultraglass," cutting off all rays excepting those of wave-lengths between 3200 and 3900 Å.U.

After examining at intervals for 8 days it was found that the most rapid germination and greatest growth was made by the seedlings illuminated through the Corning Ultraglass and for this reason it was concluded that "ultra-violet light of lesser wave-lengths appear to stimulate germination"—a conclusion opposed to that reached by Popp in 1925. On the other hand, Sheard and Higgins concluded that wave-lengths of 3200–3900 Å.U. were most effective in inducing growth subsequent to emergence of the radicle. There is no indication of any attempt to equalise the intensity of illumination or to compensate for inequality by adjusting the time of exposure in the different groups.

In the same year a publication by Delf, Ritson and Westbrook (1927) broke new ground by attempting a more intensive study of the effects of short daily exposures to the ultra-violet and other radiations of an "Ulviarc" mercury vapour lamp of the Cooper-Hewitt type (3½ amps, 150 volts on direct current). Seedlings

of *Arachis* and *Voandzeia* and *Trifolium* were chiefly used and the exposures were given at a distance usually of 2 feet for 10 minutes, 5 minutes, 2 minutes and 1 minute daily. In *Trifolium subterraneum*, irradiated from the day of planting, a delayed germination and stunted habit of the seedlings resulted. The epidermis showed signs of collapse at an early stage, the stomata succumbing first of all, and it was soon reduced on the first developed leaves to a mere brown shrivelled film. Other anatomical changes involved were seen in the lack of differentiation and greater compactness of the mesophyll of the leaves of the experimental plants. These experiments were conducted in the autumn of 1926, and, owing to the dull weather and unfavourable season, the seedlings made comparatively slow growth. The remaining irradiated seedlings all died after the conclusion of the experiment, but the control plants survived and for the next month half of them were irradiated daily for 30 seconds at a distance of 8 feet. Two months later there was a marked difference in the plants, the size and vigour of those irradiated being much greater than those of the non-irradiated plants. As there were but five in each lot these results must be regarded as suggestive rather than conclusive[1], but it is not unlikely that the detrimental effects noted for the shorter rays by all observers may be connected with over-exposure.

In experiments with cuttings of *Fuchsia*, *Coleus* and other plants retardation of flower formation and promotion of leaf-fall were conspicuous features after exposures varying from 2 to 10 minutes daily. Further experiments with *Voandzeia subterranea* were conducted, varying the length of day to which the plants were exposed. With a normal day, combined with illumination from an electric bulb all night, no appreciable effect (apart from the production of somewhat shorter and narrower leaflets) was seen either in the growth of the plants or in their response to ultra-violet light. When either normal plants or those given 24 hours' light as above were also irradiated daily for 5 minutes, over a period of about 4 weeks, a number of definite anatomical changes were observed, such as reduction in the total thickness of the lamina, development of more compact mesophyll, reduction of all mechanical tissue and collapse of the cells of the upper epidermis. With longer exposures changes occurred in the same sense but more markedly, and with shorter exposures the same type of change was observed but to a less extent. When other plants were given a shorter day by regular artificial darkening it was found that the shorter the day the more marked were the modifications in structure due to the daily irradiations with ultra-violet light, although these were only given for 2 minutes at a distance of 3 feet. Investigations were also made as to the leaf measurements and dry weights of each set of plants. Experiments along the same lines are being continued by these authors, using screened light, but the results have not yet appeared in the press. A brief preliminary report of experiments along similar lines has been made by H. R. Dane (1927), who grew soy beans under "rigidly controlled" but unspecified conditions and gave daily irradiations with a

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[1] In an attempt to repeat exactly the conditions of this experiment in the following autumn, the experimental plants were completely destroyed in one night by some greenhouse pest when at a critical stage.

quartz mercury vapour lamp. No particulars are given as to the kind of lamp, duration of exposures or distance between plant and source of light, but it was found that the stems became brittle and the internodes shorter, whilst certain anatomical changes also occurred, such as reduction in the number of medullary rays. A fuller account is promised at a later date.

The striking effects produced by the very short exposures used by Delf, Ritson and Westbrook are in marked contrast to those briefly described by Coward (1927) in the same year, in connection with the production of vitamin A in etiolated seedlings of 2 or 3 day old wheat exposed to the light of a quartz mercury vapour lamp (Cooper-Hewitt, 4·5 amps, 50 volts on alternating current). According to this author the irradiation given daily for 4 days accelerated the formation of vitamin A in the tissues, but the same effect could be observed when all the rays shorter than 3130 Å.U. and also more than half those between 3130 and 3650 Å.U. were cut off by glass screens. Therefore the acceleration could only be due to the near ultra-violet or to this together with the green and blue bands in the visible spectrum. In the course of these experiments the wheat plants were irradiated for 1 hour, 4 hours, or in some cases 8 hours, for 4 consecutive days without apparent harm to the seedlings. This is in such striking contrast to the results of Delf, Ritson and Westbrook that I took an early opportunity of consulting with the author on details of the method employed. Something of the difference may be due to the use of a less powerful source of light; something to the possibly greater resistance of the plant used, but it appears likely that the real explanation lies in the vertical habit of the leaves allowing no direct incidence of the rays, which were more or less parallel to the laminae instead of perpendicular, as is the case with dicotyledonous leaves.

Progress along other lines has been made in the attempt to utilise the properties of ultra-violet light in the cultivation of plants. These include observations as to the lethal effects of ultra-violet light on bacteria contaminating fungal cultures. However, according to Tanner and Rider (1923) yeast cells have little more resistance than the bacteria associated with them. In the experiments of Woodrow, Bailey and Fulmer (1927) irradiation of yeast cultures resulted in the development of toxicity in the medium, owing apparently to the action of the short rays on the sugar present in it. It has been stated by Pickler and Wober (1922) that ultra-violet light can be used successfully against cereal rusts, and it seems probable that the destruction of superficial parasites, such as mildews, would also be affected by irradiation not so prolonged as to give a harmful effect on the epidermis below.

Apart from artificial irradiation, other attempts have been made to utilise the ultra-violet of sunlight by the use of specially prepared glass. The pioneer work in this direction was that of Schanz (1919), who found that his plants grew better under red than under a blue glass cutting out most of the ultra-violet. With *Petunia*, *Oxalis*, cress, lettuce and other plants, the best growth appeared in those grown under "Euphos" glass, cutting out all the ultra-violet rays and those below 4200 Å.U. but transmitting red rays; with *Fuchsia*, bean and tomato, those under Euphos glass flowered earlier than those grown under ordinary glass or in the open.

Production of chlorophyll in etiolated plants of potato was favoured by red glass screens, and Schanz concluded that the ultra-violet of the solar spectrum inhibits both general development and flowering. He even recommends the use of Euphos glass for greenhouses to cut out these rays in cultivating plants.

The subsequent experiments of Popp (1926) point to a very different conclusion. Greenhouses were prepared, roofed with special glass of known transmission and screened with graded cheesecloth so that the light intensity reaching the interior of each was as nearly as possible equalised, after allowing for both the transmissibility of the glass and the distribution of energy in the different wave-lengths as received in the open from the sun on a cloudless day. Amongst the plants used were soy beans, buckwheat, tobacco, sunflower and *Coleus*. In the house roofed with "Noviol O" glass, where all the ultra-violet of the solar spectrum was cut off and only the visible and heat rays admitted, no effect could be detected in petunias, soy beans, sunflowers and other plants, excepting a slight increase in height and a somewhat earlier flowering. Thus, according to these results, although the ultra-violet rays of the sunlight are not indispensable, they are yet certainly not injurious as asserted by Schanz.

In England, experiments of this kind have not yet been carried very far, but in some cases the use of Vitaglass for greenhouses or frames appears to have been tried with success, and this method has been suggested for forcing lettuces. Two greenhouses recently constructed with Vitaglass at the Royal Gardens, Kew, may be expected to furnish an opportunity for placing such attempts on a scientific basis. It is well known that most of the ultra-violet component of such sunlight as can penetrate through the smoke and fog of industrial areas is cut off, and the rest still further diminished by the passage of the rays through ordinary window glass. Prevalence of atmospheric moisture and cloudy skies leads to the same loss to a less extent. It is therefore not unnatural to expect that greenhouse plants, and particularly stove plants or those from sunny regions, should be benefited by the use of any glass which is more transparent to the shorter rays of the solar spectrum.

It has sometimes been suggested that, since the ultra-violet of sunlight is such a variable factor in our climate, it cannot be of real significance to plant life, but no one who is familiar with the recent advances in heliotherapy would be likely to take such a view. In this connection it may be of interest to quote the results of Hess and Anderson (1927), which show that the rays of greatest potency in preventing and curing rickets are mainly of wave-lengths 2800 and 3025 Å.U., using an apparatus which has made it possible for the first time in biological work to produce monochromatic illumination of equal energy content, suitably orientated and of sufficient intensity to give the desired result. It is thus evident that in sunlight also a very narrow region of the ultra-violet (in the region of 3025–3130 Å.U.) may have a very far-reaching and for long unsuspected effect, even in regions where there is much absorption in its passage through the atmosphere.

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TISSUE CULTURE FROM THE STANDPOINT
OF GENERAL PHYSIOLOGY

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CONTENTS

INTRODUCTION.

TWENTY-ONE years have elapsed since the appearance of Harrison's paper in the *Proceedings of the Society for Experimental Biology and Medicine* (69) in which he described the successful cultivation of nervous tissue outside the organism. This investigation, besides giving a decisive answer to the very vexed question for which it was undertaken, namely, the origin of nerve fibres, opened up, by the methods which were employed, a whole new field for research, since it clearly demonstrated that metazoan tissues can not only survive, but continue their activity if placed in suitable media outside the organism. Harrison cultured small fragments of the central nervous system of frogs by suspending them in drops of lymph hanging from coverslips inverted over "cavity slides," and thus started the "hanging drop method" which has since become established as a standard tissue culture technique. It has of course been considerably modified to suit special requirements, but all methods used to-day have the same underlying principles as those which formed the basis of Harrison's work, and perhaps of all aspects of tissue culture least progress has been made in improvements of technique.

The cultivation of tissues outside the organism at once led to the solution of problems in all the biological sciences. Pathology, bacteriology, and anatomy have all reaped considerable benefits, but these can, in this review, only receive incidental mention in so far as they affect discussion on the purely physiological advances which have been rendered possible. In this connection two main fields have been investigated. These are cell structure, and the problems of metabolism and growth. This distinction is purely arbitrary and made only for the purpose of adopting some system in reviewing the great mass of new data which has come to light since the technique of tissue culture was first developed. Under cell structure will be included

purely cytological information, which though perhaps strictly anatomical is never-theless necessary for a proper study of cell function, and in the remarks on meta-bolism and growth the problem of differentiation will be discussed.

METHODS.

Before proceeding to a discussion of the results obtained in tissue culture, a brief summary of the chief technical methods employed will be given (44, 61, 66, 107, 113). As in all other biological sciences the investigator is at once faced with a whole host of difficulties, some of which appear to be well-nigh insuperable. The essence of the problem of tissue culture is to provide the cells artificially with con-ditions as nearly approximating as possible to those prevailing in the organism. Complete asepsis is the first requirement and this alone has caused some investi-gators to adopt elaborate methods for working under glass shades, or even in specially sterilised rooms in which the atmosphere is kept free from dust by the constant condensation of steam on the walls. For most work, however, using the usual culture methods, observance of ordinary precautions in the handling of instruments and the tissues, and any glassware with which they come in contact, is sufficient to procure freedom from bacterial contamination. The second difficulty arises from the necessity for keeping the culture medium fresh, and free from an accumulation of metabolites. At first sight the obvious method for the solution of this problem is by some means of continuous perfusion apparatus, but although this has been repeatedly tried (11), it has so far proved ineffective, chiefly owing to the difficulty of preventing more than the toxic metabolites from being washed away. Tissues are very sensitive to washing, which is known to affect their respiration (54), and until the reasons for this have been more fully investigated it is manifestly im-possible to supply all the essential soluble substances in correct concentrations. Thirdly, tissues are strongly stereotropic, and only grow well when in contact with some solid supporting structure. Fourthly, a limit is set to the size of tissues which will remain active in culture by the degree to which oxygen can penetrate them so as to provide the cells removed from the surface with an adequate supply. The last, but by no means the least, difficulty which may be mentioned is that of keeping the growing tissues under microscopic observation.

The simplest technique for observing the activity of tissues isolated from the organism is that frequently employed by Lewis and Lewis (87, 88). This is the hanging drop method in its simplest form, and consists of suspending from a coverslip a small fragment of tissue in a drop of culture medium (which, as usually employed by the Lewises, consists of a modification of Ringer Locke's physiological saline solution with or without the addition of chicken bouillon), over the cavity of a hollow ground slide. This method has the great advantage that the conditions are as nearly standard as it is possible to obtain them, and most of the unknown factors except those introduced by the tissues themselves can be eliminated. On the other hand, it is doubtful if true "growth" occurs under these conditions. This point will be discussed on a later page. When planted, as thus described, cells migrate out from the implant on to the surface of the coverslip, or on to the air-medium interface, so that in either position,

especially the former, they are in excellent arrangement for direct microscopical observation, even under the highest magnifications. The cells are, however, probably abnormally flattened. In this very simple medium the cells die in a few days, or weeks at the longest, even if the fluid be frequently renewed, and it was not until Carrel and Ebeling worked with a medium consisting of plasma and an extract of embryonic tissues (22, 23, 47) that the problem of keeping the cells alive indefinitely was solved. By frequent subculturing of the hanging drop cultures into fresh "embryo extract" and plasma tissues have been kept alive and continuously proliferating for many years (25, 50, 51), and there appears no reason why they should ever stop. They are potentially immortal. The drawback to this technique is the immense routine labour required for renewing the medium of the cultures every forty-eight hours, and to obviate this Carrel developed the flask technique (32, 33). The essential of this is the duality of the medium consisting of a semi-solid part which is spread out on the bottom of a flask, and in which the tissues are embedded, and a fluid part which contains the nutritive materials and can be renewed daily or as often as is necessary, with comparatively little trouble. The flasks are sealed off with cotton-wool plugs in the ordinary bacteriological manner. The method has the disadvantage that direct microscopical examination of the cells is difficult, although this has to some extent been overcome by cutting a circular hole in the bottom of the flask and sealing over this a coverslip or mica slide against which the tissues are planted and through which they can, by inverting the flask, be examined. Coagulated plasma usually forms the supporting element, or solid part of the medium, although a variety of substances will serve, as, for example, cotton-wool, glass-wool, spider's web, etc. (61, 70). Such substitutes for plasma are, however, never so effective. The advantages of this technique are great. The making of repeated subcultures is less necessary, and the cells remain healthy and undisturbed for very much longer periods. The medium, owing to its larger volume, keeps "fresh" longer, and metabolites do not accumulate so rapidly, and generally the environment of the tissue remains more uniform. Moreover, several pieces of tissue can be cultured in the same flask, and hence an investigation of the chemical changes in the medium caused by the tissues can be carried out more readily, since greater quantities of material can be dealt with, and the consequences of individual variations in the tissues minimised. This method should prove useful in opening up many new lines for research.

A third method of culture is to embed the tissues in clotted plasma or other similar medium in small tubes, in a manner similar to the tube method of bacterial cultivation. For some purposes this technique has great advantages and further mention of it will be made in connection with the problems of differentiation. A few subcultures may be made, but the outgrowing cells are generally lost, so that it is really only useful for studying the changes which occur within the tissues of the implant. Again, direct microscopical observation is limited, and fixing, staining and sectioning methods have to be employed for detailed observations.

CELL STRUCTURE.

The fact that the cells tend to migrate out on to a solid surface, due to some form of stereotropism, which is possibly brought about by their mere stickiness (85, 93, 96) or merely in obedience to the laws of surface tension (55), renders them peculiarly well adapted for cytological investigation, since the surface of a coverslip forms an ideal field for their activity. And in this position the cells can be examined, whole, alive, and without subjection to the always more or less harmful processes of fixation. Consequently a much more accurate picture can now be obtained of the structure of the living tissue cell than was ever previously possible.

Optically, the normal tissue culture cell, such, as the mesenchyme which migrates from fresh cultures of chick heart in any suitable medium, shows, apart from the nucleus, very little structure (94, 95). Tissue culture cells can be readily studied for long periods under the highest magnifications with transmitted light, and for some time under dark ground illumination. Under the latter, however, observations cannot be very prolonged since the cells start to degenerate. This may be due to one of several conditions, such as lack of oxygen since the tissues have to be grown between two flat coverslips with no air space between them, or the direct influence of the intense radiation to which they are subjected, for the necessary illumination is very intense, and further, unless very carefully screened, the heat rays are sufficient to cause a considerable rise in temperature at the focus where the tissue must be placed. Even if screened from heat there is the possibility of changes caused actually by the light. Such changes have been recorded with light of much less intensity both for leucocytes and fibroblasts, but they were probably related in some way to the influence of the light on the haemoglobin which was accidently introduced into the cultures in erythrocytes, since in the case of fibroblasts the light produced no reaction unless erythrocytes were present in the culture medium (46). Maximow (101) records the direct action of light as being a method of causing polyblasts in culture to withdraw their processes and round off. Nevertheless the difficulties of dark ground illumination can to some extent be surmounted, and much valuable information has been obtained by its use.

The *cytoplasm* varies in consistency but is always more or less fluid, and contains no signs of a network nor any of the other structures which have been described and must therefore now be considered as artifacts produced by fixation and staining (95). This does not necessarily detract from the value of fixing and staining methods since under any given set of conditions the results produced by the same reagents will always be the same for a given type of cell, so that different cell types will constantly produce definite histological pictures although sometimes these may have but little connection with the actual structures present in the cells when alive. The cytoplasm of tissue culture cells then is to be regarded as a fluid, showing no structure under direct or dark ground illumination. There is no visible cell membrane as such, but merely a line of demarcation between cytoplasm and medium owing to their different optical densities. The dark field, as would be expected, shows this junction as a faint bright line. Between some cells, particularly those forming membranes, there

generally appears an optically denser separation area whose properties have not yet been sufficiently studied. In the cytoplasm there are regularly to be found definite structures which fall into three categories. First, mitochondria are of almost universal occurrence. They have been demonstrated by Lewis (92) both by vital staining with Janus Green B, for which stain they show a ready affinity, and by direct observation under dark ground illumination. Unfortunately the stain is rather toxic and the cells do not live long after treatment. The mitochondria are normally thin rod-like bodies, which are constantly in a state of wriggling motion. This can be beautifully seen by means of the dark ground illumination. When the cell is in any way unhealthy the mitochondria show it by rounding off, even swelling up into vesicles under certain conditions, such as immersion in an acid medium. During mitosis the mitochondria shorten. Their function has been a subject for considerable discussion(105 a), and still remains obscure. At one time they were considered to be symbiotic bacteria (43), then to be concerned with the manufacture of secretions, and now perhaps the prevalent view is that they are concerned with oxidations in the cell. This seems the most reasonable view and has some evidence in its favour. Firstly, the Janus Green B stain is slowly turned pink, and finally bleached to the leucobase. Secondly, if the deeply stained (Janus Green B) cell is placed in a solution containing potassium cyanide the mitochondria are at once bleached. This does not occur when the cells are placed in acid media, indicating that the potassium cyanide as usual has affected the oxidations of the cell and the Janus Green B is immediately reduced. The colour returns again on restoration to a normal medium. The mitochondria appear to be of a lipoid nature and substances such as alcohol, acetic acid, and so on, which attack lipoids, reduce oxidations in the cell. Kakiuchi (78) has measured the respiration of heart tissue after treatment with various reagents and finds that acetic acid and alcohol reduce the respiration almost to zero, and histological examination shows that these are the particular reagents which destroy the mitochondria. But this does not necessarily mean that the mitochondria are the special seat of the cell oxidations, and all the evidence in favour of this hypothesis is circumstantial rather than direct. We must therefore still regard the function of mitochondria as an unknown quantity.

The second category of cytoplasmic inclusions consists of the granules and vacuoles which may or may not be present. It has always been assumed that in the healthy embryonic mesenchyme cell these structures are almost entirely absent (95); and it is certainly true that under the abnormal conditions of culture they increase greatly in numbers, and moreover their appearance depends largely on the medium employed. Lewis (90) has shown that those which stain with neutral red tend to disappear under the influence of glucose. Similarly Carrel and Baker (39) find that cultures growing in pure proteoses obtained from fibrin digests show fewer cell inclusions of this type than cultures growing in extract of embryonic tissues. Vacuoles which stain with neutral red are of fairly general occurrence. Their colour varies slightly according to the pH of the medium, but is rather difficult to alter by this method (95). A relatively strong solution of potassium cyanide bleaches them but not irreversibly (95). Nothing definite can be stated as to their function. Dark ground

illumination sometimes reveals Brownian movement occurring among the small vacuoles, indicating the fluidity of the cytoplasm (95).

The third category consists of the fat globules. These again are absent, or nearly so from young healthy culture cells, but increase rapidly in various media, also in cells which are commencing to show degenerative changes.

The Golgi apparatus has until recently not received much attention from tissue culturists, and although a specialised area of cytoplasm is visible near the nucleus, direct evidence of its existence in the living cell is lacking. Parat, using intravital stains on various kinds of tissues, identifies the Golgi apparatus with certain structures which stain with neutral red. This work has been recently reviewed by Nath (105 a).

The usual staining methods produce, according to Ludford (98), typical pictures of Golgi bodies in those cells of a tissue culture which are near the edge of the central mass, but as the cells migrate away from the implant and become flattened against the coverslip the Golgi apparatus undergoes significant changes. It separates out into thread-like structures which later break up into globules, thus giving a quite different appearance from the usual Golgi apparatus. Ludford connects these globules, and in fact the whole mechanism with the capacity of the cell for fat storage and metabolism.

The *nucleus* again in culture cells is optically almost structureless, and the nuclear membrane appears merely as an interface between cytoplasm and nucleoplasm. Linin threads and similar structures are products of fixation and staining, but one or more nucleoli can always be observed as they are slightly more opaque than the typical nucleoplasm. Under close observation the nucleoli are seen to change shape, and dark field illumination indicates that they have a coarser consistency than the nucleoplasm (95).

Mitosis has received considerable study by workers on tissue culture (83), particularly by the late T. S. P. Strangeways (112), who described the process in detail, giving the average times occupied by each phase of the division. He also drew attention to the rounding off of the cell during the early stages of mitosis, and the curious bubbling movements on its surface as the chromosomes are separating towards the two daughter poles. The chromosomes are distinctly visible owing to their different refractive index. The essentially dynamic character of mitosis has recently been strongly emphasised by the work of Canti with the cinematograph as recorder; and incidentally this method of observation has already led to many other interesting and important results. Strangeways found that the average time for complete cell division in chick cultures at body temperature was in the neighbourhood of thirty-five minutes, although different cells varied from twenty-three to sixty-five minutes, and it is very interesting to note that this average is in very good agreement with similar figures given by Lambert (83), Levi (86) and Lewis and Lewis (89). Levi and the Lewises observed cells growing in plasma media, and in Locke-Lewis saline solutions respectively. Strangeways used a plasma and embryo extract medium. This clearly indicates that the actual process of cell division is largely independent of the external medium, and that any effects caused by the latter must chiefly concern

the growth of the cell and the interkinetic period. This distinction between growth and cell division has been strongly emphasised by Gray (68 *a*) in his work on segmenting echinoderms. The early stages of segmentation are concerned with the subdivision of the existing cytoplasm, and very little if any growth is occurring. During this process cell division occurs automatically and is probably only affected by the surrounding medium in so far as this alters the rate of growth of the asters. Mitosis itself has no effect on metabolism, and the results mentioned above suggest that the metabolism of the cell has no effect on the actual mitotic process, but only on the intervening periods.

So far there is no evidence as to whether the interkinetic period is variable. Presumably it is, but direct evidence should shortly be forthcoming when the cinematograph is applied to this investigation. Fischer (61, 62) has demonstrated that the numbers of mitoses occurring in a culture show a distinct periodicity, rising to a maximum about every twelve hours. The independence of the dividing cell with regard to external agents is shown by the work of Strangeways and Hopwood (118) and Canti and Donaldson (19), who investigated the effects of X-rays and radium on the growth of cells *in vitro*. Both types of radiation cause a cessation of cell division in the cultures, but those cells actually undergoing division when subjected to the rays produce two normal daughter cells. Just prior to, and in the early stages of, division the cells are very sensitive to external conditions, but once the process has been initiated it proceeds irrevocably forwards. Abnormal cells are not uncommon in tissue cultures, especially when the medium is old or unfavourable. The normal nucleo-plasmatic ratio is disturbed, and bi- or multi-nuclear cells make their appearance. Often giant cells occur which may contain considerable numbers of nuclei. Strangeways (115) has described three ways in which this may take place. First, the nucleus divides by typical mitosis, but nuclear division is not followed by cytoplasmic division. Second, a typical mitosis may take place but the cell divides unequally so that a small portion of cytoplasm only is nipped off and a binucleate cell remains. The cinematograph film made by Canti shows an occurrence similar to this. In the third type described by Strangeways the mitosis may proceed normally as far as the metaphase, and then in the formation of the new cells multiple nuclei make their appearance, and the cell may or may not divide. Lambert (83) and Lewis and Lewis (95) describe atypical mitoses as occurring in cancer and normal cells, and multipolar mitoses of various types have been noticed.

Giant cells have also been investigated by several workers (8) and have been produced artificially. Barta correlates their appearance with the amount of oxygen available for the cells. He cultured several explants from rabbit lymph nodes in plasma and tilted the slide in such a way as to cause the medium to be thicker at one end than at the other, and found that the explants behave differently according to their positions. On the surface, growth was normal and fibroblasts, lymphocytes, and reticular cells all made their appearance, but with increased depth the cytoplasm hypertrophied, fat drops appeared in greater numbers, amitotic nuclear division occurred without division of the cytoplasm, and neighbouring cells fused. As a result of these last two processes giant cells were produced. These changes occurred

when the distance from the surface was greater than 0·5 mm., and Barta suggests that deficient oxidation is the primary cause. It is interesting to note that the addition of an extract of embryonic tissues partially counteracted these changes.

Similarly Lambert[82] produced giant cells from leucocytes by the addition of foreign bodies such as lycopodium powder and he points out that in certain cases irregularities on the coverslip may act as foreign bodies in this respect, and form centres for giant cell formation.

It is now established that amitosis is a definite phenomenon but probably one which only occurs in unhealthy and degenerating conditions of the cells[95]. In fixed preparations it has been described by numerous authors, but has very rarely been seen by direct observation in the living cells.

One of the problems which has recently received considerable attention is the relationship which exists between the cell and its neighbours. Several methods have been used to isolate individual cells, such as protecting them with small globules of mercury and killing all the rest with ultra-violet light[6, 44], or growing a culture together with minute fragments of cotton wool[59], and pipetting off fragments to which single cells were attached, but so far, although the cells may survive for a short time, growth and division have never been observed, and generally the cells round off and remain completely inactive[61]. It is only when cells are grouped together that they manifest their normal activities, so that in this respect it seems necessary always to regard the metazoan cell as forming part of a tissue, and being unable to function when isolated. This at once raises the questions as to why isolated metazoan cells should thus fail, when protozoa can exist by themselves, and produce colonies from single individuals[108], and secondly what is the nature of the relationship between the cells in a normal tissue? Some authors[61, 64] consider that actual protoplasmic connections are made between the cells, while others prefer the view that substances slowly diffuse from one cell to another. Protoplasmic connections occur freely between the cells of plants, so that there seems no *a priori* reason that they should not also exist in animals but on the whole the evidence seems to be rather to the contrary. Fischer[60, 61] has endeavoured to establish the fact by studying the pulsations of two fragments of heart grown close together in the same medium, and has found that as long as no cells bridge the gulf between the fragments they contract independently, but that as soon as the fibroblasts from each explant have intermingled there is at once a disturbance of the previous rhythms, the contractions become irregular and often fibrillar. When the distance between the fragments is smaller, the fusion between them takes place more quickly and completely, so that they then begin to contract synchronously; sometimes this does not occur; and on those occasions histological observation shows the presence of a sheath of connective tissue between the fragments. Also fragments from duck embryo heart were grown adjacent to chick explants and although they proliferated and contracted regularly in the mixture of chick plasma and embryo extract, which was used as a medium, the rhythms never became synchronous. Fischer brings forward this evidence to demonstrate the existence of direct protoplasmic connections between the cells. It demonstrates a physiological interdependence of the

cell types, but does not unequivocally show that morphological connections exist; and the method seems to be open to objections on other grounds also. Chambers (40) has brought some interesting work to bear on this point. He found that often, immediately after cell division, a slight visible protoplasmic connection remained temporarily connecting the two daughter cells, then, when one of the cells was injured so that the nucleus degenerated, immediately the other cell showed similar changes. On the other hand, when he injured one of two cells which were so closely in contact that no visible line of demarcation separated them, the injured cell immediately withdrew, and degeneration was confined to that cell, clearly indicating that the cells were distinctly separate units. In any case, from the recent work of Canti, as illustrated in his cinematograph film, it is evident that if intercellular protoplasmic bridges exist they must be secondarily developed, since immediately after mitosis the daughter cells can be seen to travel widely apart, as if repelled from one another. Levi (85) has suggested that cells may come into immediate contact with one another and unite by their innate "stickiness," thus forming membranes and tissues. He reports having observed two cells to come together with processes actually fusing, a passage of mitochondria and granules from one to the other, and then a complete separation; he develops the idea that cell boundaries have comparatively little significance, but that there are always cell territories, each one of which is presided over by a nucleus, and that the cytoplasmic boundaries may disappear in the formation of syncytia which may only be temporary, or more or less permanent, according to circumstances.

For many fields of investigation pure cultures of cells are necessary or desirable, and now techniques have been elaborated by which several types of cells can be cultured in the pure state. The success of the technique depends largely on obtaining exactly the right conditions of the medium. Pure cultures may be obtained in one of two ways. They may be started pure, by explanting tissues of one type only, as can be done for epithelial cells, cartilage, and thyroid (a number of cells must be explanted for the reason mentioned above), or they may be started mixed and by continuous proliferation one type may grow faster and better and ultimately eliminate all other types. This has been successful in the case of fibroblasts, but is apt to be a dangerous method on account of the fact that the microscopical appearance of cells changes during culture and, optically, two different cell types may become indistinguishable. For example, epithelial cells form membranes when growing on the surface of a coagulum, but when they penetrate below the surface they become fusiform or spindle shaped and are easily confused with fibroblasts (121). Even so, however, they are usually distinguishable from each other by staining methods. (Van Gieson for epithelium and connective tissue. Fischer (49, 61).) So far fibroblasts (23, 24, 25, 26, 50), epithelium (51, 56), cartilage (57), thyroid (53) and leucocytes (27) are among the normal tissues which have been successfully cultivated *in vitro*, for a period of time considerably in excess of that occupied by mere survival, although as yet they cannot all be kept alive indefinitely, as can fibroblasts. Differential susceptibility to poisons has also been used as a method for obtaining pure cultures. For example, the action of arsenious oxide is more violent on

fibroblasts than on macrophages (38). Similarly Krontowski and Radzimovska find that fibroblasts are more resistant to treatment with acid than are other cell types, and suggest this as a method for obtaining pure cultures (79).

Two problems at once arise from the study of cells in pure culture. Firstly, are cell types interconvertible, and, if so, to what extent, and secondly, are cells in culture to be considered as de-differentiated? And these problems are of course intimately connected. A considerable amount of work has been done especially on the second of these questions, and Champy (41) maintains that in culture the cells revert to an embryonic type, losing all characteristics of the type from which they arise. Certainly from microscopical observation there is much that can be said in favour of this view, since in culture the various tissues may be very difficult to distinguish by mere inspection. Champy also produces evidence showing that gland cells in culture fail to produce their characteristic secretions (42). But bricks cannot be made without straw, and does the culture medium provide everything that is provided by a continuously circulating blood stream? Barta (7) seems to come nearer the truth when he considers that the cells observed in cultures, although they have lost their characteristic structures, are nevertheless still true to type, but have adapted themselves to the abnormal conditions of the medium. For example, as already mentioned, Uhlenhuth (121) and others have shown that epithelial cells form a membrane on the surface of the coverslip or coagulum, but when they penetrate the latter they become spindle-shaped and difficult to distinguish microscopically from fibroblasts. Again, Ebeling and Fischer (49) have been able to obtain characteristic staining reactions from epithelial and connective tissue cells which had grown together for several weeks. At best, the tissue culture medium is very abnormal, and the cells tend to adapt themselves, so that it is only to be expected that they will appear and behave abnormally, although they still have the potentiality for once again becoming typical both in appearance and function. This has been demonstrated by the fact that under "unfavourable" conditions of the medium keratinisation of the epithelium takes place (51, 61), and Drew (45) has shown also that the presence of fibroblasts in the vicinity of the epithelial cells will produce tubule formation in kidney epithelium, and keratinisation in other types. Again, under certain conditions, iris epithelium has produced pigment (51, 61) and pure cultures of thyroid cells have produced "colloid" (53). A reasonable view therefore seems to be that cells in culture adapt themselves to the medium, often with temporary loss of their characteristic structure, but they still remain fundamentally true to type, not de-differentiating to an indifferent tissue, so that given the appropriate medium they might be expected to regain their full functions.

Quae cum ita sint, it does not seem probable that the cell types should be interconvertible and there is no evidence, for example, that fibroblasts become epithelium or *vice versa*. Nor is there evidence that any other well-marked tissue has changed its essential characteristics for those of a different group. There is, however, one exception to this. Carrel and Ebeling (27) have brought forward evidence that the leucocytes of chicken plasma during prolonged cultivation first become one and all of the large mononuclear type, and that occasionally these become converted

into fibroblast-like cells, with the characteristic staining properties of the latter. This is interesting in that in the same paper and elsewhere (38) these authors illustrate the different behaviour of these two types of cell when cultured. Monocytes always remain isolated and they survive well in serum, whereas fibroblasts tend to remain together, and serum for them forms an inadequate medium. Colonies of leucocytes keep apart, while those of fibroblasts tend to fuse. Fischer (63) has brought forward similar evidence that leucocytes can become fibroblasts. He, however, added to the culture medium small pieces of dead muscle which the leucocytes phagocytised, and it is possible that a contamination by fibroblasts might have occurred. His control experiments indicated that this was unlikely, but the possibility was not altogether ruled out. After several passages into fresh media fibroblast-like cells appeared. The dead muscle ceased to be attacked, and meanwhile an almost typical connective tissue type of growth occurred. Maximow (100, 101) and Bloom (9, 10) have recently published results of similar experiments which produce striking confirmation of the results of Carrel, Ebeling and Fischer, and indicate very clearly the extremely plastic nature of the leucocytes. Fischer (67) has now brought forward evidence to show that fibroblasts can become converted into macrophages, which is the first stage in the reverse process.

When the various difficulties of the technique of tissue culture have been further overcome, the method should prove very useful in experiments into the intimate nature of such processes as secretion, or muscular activity, or the response of re-acting cells to doses of drugs, or other changes in the medium. But, as previously indicated, owing to the inadequacy of the medium and general conditions of culture, very few cells have up to the present been observed in their functional condition. Among such, however, may be mentioned heart muscle. Several observers (12, 81) have described the growth of heart muscle, some of them probably erroneously. Lewis and Lewis (95) conclude that heart muscle cells migrate out from chick embryo cultures comparatively rarely, and then generally only from very young embryos between the third and fourth days of cultivation. Isolated cells have been observed contracting rhythmically, thus conclusively establishing the myogenic origin of the chick heart beat. These contractions have taken place entirely in the absence of nerves, and with rhythms which may vary from cell to cell. Fibrillae are believed by Lewis to be artifacts of fixation, and the only things corresponding to them in the living cells are tension striae. Cross striations occasionally occur, but are apparently not a necessity for successful rhythmical contraction, and are not as frequent as would be expected considering that the muscle cells of the explant are all striated. This has been taken by some as a point in favour of Champy's contention that cells in culture tend to de-differentiate. Finally, Lewis and Lewis (95) emphasise the considerable quantity of glycogen which is generally present in outgrowing muscle cells.

Gland cells have to some extent been studied *in vitro* but perhaps not as fully as might be expected. Thyroid (53) has been cultured in a pure state, and well illustrates how dependent the cells are upon the conditions of culture. It has been cultured on the surface of fibrin clots with a medium containing embryo extract. As long as the cells remain on the surface they behave as pavement

epithelium, but sometimes they grow down into the coagulum and then form acini, which as previously mentioned are capable of secreting the typical "colloid" of the thyroid. Champy (42) cultured the prostate gland of the guinea-pig which normally produces a secretion which in minute concentration coagulates the contents of the vesiculae seminales. He found that the secretion no longer occurred *in vitro*, so that the fluid from a two-day culture only produced a weak reaction, while no reaction took place after four days. He found also that the tissues kept on ice for four days had lost all of their ferment. It might be expected that cells on ice would lose their ferment slower than those kept at body temperature, so that it is possible that the cultures had actually secreted some ferment during their four days' growth. In any case the medium in all probability did not contain any excess of such materials as are necessary for the manufacture of that particular secretion. It seems possible that in the future much interesting work may be done by the aid of tissue culture methods on the elucidation of the problems of secretion and the manufacture of ferments, but before this can take place the conditions under which cells can function will have to receive more careful attention. As will be shown again later, it is necessary to distinguish, with Thomson (120), cultures which show uncontrolled growth from those which show somatic or organotypic growth, where the normal differentiation is more complete, and the cells are capable of behaving more as they do in the body. Similarly much might be learnt concerning the action of drugs and alterations in the medium on isolated but functioning muscle cells. As the result of some experiments as yet unpublished the author has shown that cultures of the intestine of the ten-day-old chick embryo grow better and survive longer when cane sugar is added to the medium; cultures of the chick heart do not behave in the same way. They receive no benefit from this addition to their diet, so that the evidence thus far favours the view that the intestine even at this age contains invertase or some other similar ferment which is capable of rendering the cane sugar utilisable by the tissues. Several problems are suggested by this experiment. In the first place the method might be used for investigating the time at which the various enzymes first make their appearance in the intestinal and other glands; and secondly it would be interesting to investigate by some form of continuous culture method for how long the intestine would go on producing the invertase under the conditions of culture, and also if possible, to ascertain what particular substances are necessary in the medium for its production. This again might be applied to a study of the pancreas and other glands producing digestive ferments, the products of whose action might be expected to assist in the growth of the tissue cultivated, or other tissue placed in the same culture.

The wandering cells of the body have received considerable attention from workers on tissue culture, and in this respect some of the lower animals have been subjects for investigation, and have, as might be expected, proved to be of considerable interest. Leo Loeb (97) has studied the amoebocytes of *Limulus*, both from the point of view of their mechanism of movement, and also their capacity for the formation of a definite tissue. Besides this, as already mentioned, Carrel (27, 28, 35, 36) and his school have cultured chick leucocytes pure, and find them rather more difficult to

maintain in an active state than fibroblasts, but as a result of this work a new light has been thrown on their normal functions in the body. Previously it was supposed that their function was one of scavenging the body of bacteria, necrotic cells, and other debris and foreign bodies. Carrel, however, has found that they can live well in plasma, where fibroblasts fail, and that apparently they can not only utilise the substances in the plasma for their own use, but can hand on to the fibroblasts food substances which the fibroblasts cannot obtain for themselves, except in such media as embryo extract. Carrel has designated these food substances produced by the leucocytes as trephones. Further discussion on their properties will be reserved till a later page, but it may be mentioned here that in this way leucocytes may play a very important part in wound healing, not only in "clearing up the mess," but in actually stimulating the fibroblasts in the immediate vicinity into renewed proliferative activity.

On a similar subject, namely, on the method of phagocytosis occurring in the lungs, Carleton (21) has produced much interesting information and has used the capacity of tissues to continue to function *in vitro* for an investigation of the parts played by the various tissues in this process. He concludes that *in vitro*, the cells of the alveoli are actively phagocytic for coal and carmine particles, whereas he found no evidence to show that the endothelial cells of the blood vessels had this capacity. He made use of the tube technique for the cultures and the method has since been widely used for special purposes. As already described it consists in suspending the tissues in clots of plasma, and embryo juice if required, formed in very small tubes of little more than 1 c.c. capacity. The tissues may be repeatedly subcultured, though with the loss of the cells which have migrated out into the medium.

For further information on the behaviour of cells in cultures the reader is recommended to consult the article by W. H. and M. R. Lewis in *General Cytology* (95).

METABOLISM AND GROWTH.

We may now pass to a review of some of the work which has been done on the metabolism and behaviour of cells in culture, and to a consideration of tissue culture as a method for studying such metabolism and for elucidating the problem of growth. As already pointed out, growth in culture may come under two headings, uncontrolled and somatic; the latter will be dealt with later, and at present remarks will be confined to the former. The term "uncontrolled" growth has been applied to that very active cell proliferation which is typically seen in hanging drop cultures of embryonic tissues explanted into suitable media such as embryo extract, and it is this type which is now under discussion.

Moreover cells explanted under tissue culture conditions may behave in any one of several ways, which will depend partly on themselves and partly on the medium in which they are planted. In the term medium is included not only the chemical constitution of the surrounding fluid or coagulum, but also its physical and mechanical properties, as these all play their part in influencing cell behaviour. In choosing the tissue and medium for culture two main problems are encountered; to obtain a medium as nearly akin as possible to conditions reigning in the body, and to

eliminate as many unknown factors as possible. By the very nature of living material these two are mutually antagonistic and some form of compromise has to be struck. For most work a standard tissue has now been adopted, namely, a pure culture of fibroblasts, or failing this, chick embryo heart, which although by no means a pure tissue, nevertheless has many advantages in that it is easy to obtain, grows well and fairly constantly, and gives rise mainly to fibroblasts. Pure cultures of various other types of cells have also been used for special problems, but, except where stated to the contrary, it will be assumed that the tissue under discussion in the following pages is either pure fibroblasts or chick heart tissue.

If tissues are explanted into hanging drop cultures with a saline fluid such as Ringer solution for medium, then, granted that the various physical necessities as to hydrogen ion concentration, osmotic pressure, temperature and the like are provided for, they will survive for a few days or perhaps longer. If the tissue is derived from an adult animal then practically no activity will be observed; perhaps after a considerable latent period a few cells may migrate out on to the coverslip, or on to the air-fluid interface. If the tissue is fixed, stained and sectioned after a few days, some internal reorganisation will probably be manifest, but essentially the condition has been one of mere survival and in that sense not really tissue culture. If embryonic tissues are placed in the same medium, the sequence of events is fundamentally the same, except that the latent period is shorter and more cells migrate out. All the energy and materials utilised in the process are of course derived entirely from the reserves present in the tissues themselves.

Possibly, where the oxygen supply in the centre of the fragment is low, necrosis may set in, and this may lead to the liberation of food substances. Often quite a large area of newly formed tissue may appear round cultures in saline, but since mitosis is of comparatively rare occurrence, most of it is probably due simply to emigration of cells originally in the explant. Actual migration has been watched in epithelium of the frog(99), also by the cinematograph in chick fibroblasts. Cells cannot be kept for more than a few days in this condition, and soon round off and die.

A very similar state of affairs arises in cultures in coagulated plasma media. Plasma probably has not much in the way of nutritive substances to offer to fibroblasts, and does not therefore greatly aid in lengthening the life or increasing the activity of these cells, except for the very important fact that the fibrin network, which forms on coagulation, provides the best supporting structure on which the cells will grow. Tissue cells are strongly stereotropic and they always tend to emigrate more easily on to any solid structure. In this way cultures of fibroblasts are apparently more active in plasma than in simple saline fluids. Some cells, such as leucocytes, to which reference was made above, can utilise the substances present in plasma as a food material. This is often accompanied by liquefaction of the plasma. But, apart from leucocytes, tumour cells and one or two other types, cells in culture only utilise the plasma as a scaffolding, and cotton wool, spider's web, etc., will answer this purpose almost equally well.

There are however many substances which may be added to the saline, or plasma medium which bring about a considerable increase in the activity of tissues planted

therein. The most satisfactory and apparently complete is embryo extract, which is simply a saline extract of any embryonic tissues, not necessarily of the same species as the tissues to be cultured. For example, duck fibroblasts grow well in chicken embryo extract (60), and the differences between the two sources of tissue may be even wider than that (29), for rat tissues will also grow in chicken extracts (104), and rabbit embryo extracts stimulate chicken tissues (34). In such a medium the tissues at once start to proliferate actively, and the size of the tissue increases by the addition of new protoplasm. If plasma is present also in the medium, it forms a support for the tissues, which can then be divided, washed, and replanted in fresh medium. If this is done regularly every few days, so that the tissue is always kept small enough to receive an adequate oxygen supply throughout, and noxious metabolites are never allowed to accumulate, it may be not only kept alive but continuously growing for an indefinite time. In this condition the cells migrate out actively and cell division by mitosis is abundantly observed.

Such uncontrolled growth at once raises a variety of problems, few of which have as yet been solved. The first question that naturally arises is concerning the nature of the factor or factors which are responsible for the activity of embryo extract. Does embryo extract act merely as a complete diet for the tissues, or does it contain a special stimulant for cell division, or stimulant for migration, or one which, like thyroxin, increases metabolism generally? Is it possible to stimulate cell activity without also stimulating division, and *vice versa*? In order to attempt to answer these questions it is necessary to enquire into the conditions present in a tissue culture, and to study the influence of various media on the behaviour of the cells.

The influence of inorganic salts in the medium has been subjected to considerable study by several authors (72, 87, 88, 95, 126), but as might be expected they do not prove to be any more than controlling factors which limit growth if present in abnormal concentrations. As yet there is no evidence that any of them has any stimulating action; and, as has already been pointed out, cultures planted in media containing nothing but inorganic salts are really only in a state of survival, living on their own reserves. The influence of small traces of iron or iodine or other inorganic bodies of physiological importance has not been extensively investigated. From the connection between iron and such a universally important substance as cytochrome, it is possible that iron administered in the correct way might have considerable effects on cell metabolism. Possibly also this applies to copper.

Inorganic bodies alone then, on the evidence so far produced, cannot be considered as directly influencing the rate of growth of cells in culture provided they are there in approximately the same concentrations as in the blood of the animal whose tissues are being cultured. Any other concentrations tend to be adverse rather than beneficial. Under inorganic bodies oxygen is not included, as this will have to receive special consideration. The concentration of inorganic salts will, however, control the effective osmotic pressure of the medium, and this does appear to have some influence on the rate of migration of cells in culture (72, 126), but has not been found to alter the amount of cell division in such cultures (48, 84). Hypotonic solutions tend

to increase migration, and cause some enlargement of the cytoplasm of the cells, while cells in hypertonic media migrate less rapidly, but more steadily and normally. Ebeling[48], however, has shown by the use of the subculturing method that only isotonic media allow of indefinite survival, so that this again illustrates the point already mentioned that inorganic salts seem to be present in optimal concentration in the blood serum, and any alterations seem to lead only to detrimental results. The same probably applies to other factors in the physical constitution of the medium. For example, for the tissues of warm-blooded animals body temperature is probably the most suitable for obtaining steady normal growth, although the tissues are not greatly injured by lower temperatures[65]. Reactions are then slowed down but no damage is sustained by the cells. Tissues will, in fact, grow well after several days in the ice chest, and cultures of chicken heart tissue are not injured by being kept at room temperature for several days; at least on returning to the incubator they quickly recover their normal rhythmic contractions.

Surface tension is a physical factor in the make-up of the medium which has not received sufficient attention, and may play a large rôle in affecting the migration of the cells on to their supporting structures, and influencing the stereotropic qualities, but again mere lowering or raising of surface tension will not determine whether cells grow or not. It is, however, interesting that most media which allow of active growth do seem to have low surface tensions as indicated by their behaviour in spreading on glass.

The last of the physical or physico-chemical factors of the medium is probably by far the most important, and that is the hydrogen-ion concentration, and not only is it the most important, but also the most difficult of investigation, and varying results are obtained according to the technique which is adopted. For example, Lewis and Felton[91] find that, in their Locke-bouillon-dextrose medium, growth can occur between pH 5·5 and 9·0, with an optimum about 6·8, and cultures that are growing well tend to bring the hydrogen-ion concentration to the neutral point pH 7·0. In a pure Ringer solution cultures even tend to become alkaline, but if glucose is present then the reverse tendency is observed, and as will be shown later glucose is definitely used by tissues in culture, so that this production of acid may be correlated with the conversion of glucose into lactic acid or CO_2. If the concentration of glucose is large, then the cultures are found to become acid, but the tissues can keep the medium almost neutral in weaker solutions. There seems to be considerable latitude therefore as to the degree of acidity or alkalinity which the tissues are able to stand, which is no doubt partly to be explained by their capacity to bring the reaction to the neutral point. Fischer[61] found that in plasma and embryo juice the optimum pH for prolonged growth was between 7·0 and 7·8. The range in these experiments was limited owing to interference with the coagulation of the plasma, but a pH below 6·0 and above 8·0 quickly proved toxic, and between these limits growth was at a maximum at pH 7·4. Fischer was, of course, using the subculturing method in which the tissue has to reach equilibrium with a new medium every forty-eight hours. This periodical adjustment on the part of the tissues may indicate why results vary in regard to the optimum pH value for growth according to the

methods employed, as it may call heavily on the alkaline reserve of the tissues or on their acid-producing capacities and so alter their whole metabolism.

The new flask technique of Carrel (32) might well be employed for testing the influence of changes in the hydrogen-ion concentration, since here the tissue can remain in the same medium undisturbed for much longer periods, with a medium whose constitution remains far more constant and incapable of being much altered by the tissues. Similar remarks might be equally well applied to Ebeling's experiments (48) on the influence of changes in osmotic pressure. The results of Fischer are interesting when compared with those of Mendeleeff (102), who grew tissues in plasma of different hydrogen-ion concentrations obtained by injecting foreign proteins into the animal some time before taking the blood. With such methods she found that at pH 5·8–6·0 the plasma is far more suitable to growth than when neutral. One wonders, however, how far this is purely an effect of hydrogen ions, and how far it is due to other factors which would also be altered in plasma treated in this way, especially when in the same paper it is stated that plasma acidified to pH 6·6 by means of hydrochloric acid, becomes toxic. On the other hand, she has taken measurements of the hydrogen-ion concentration of the blood of the adult and embryo guinea-pig (103), and has found that whereas the blood of the adult was at pH 7·4, that of the embryo *in utero* was at pH 5·8, and as the embryo grew older the pH gradually rose, till at birth it was 6·2 and six days after birth stood at 7·0. Compared with the hydrogen-ion concentration of extracts of chicken embryo tissues (pH 6·8–7·2) (61) these figures seem low. The results are therefore difficult to interpret, and apart from them, the optimum hydrogen-ion concentrations seems to be that of neutrality or thereabouts, and any deviation leads to adverse effects. Like many of the other necessities of the culture medium so far discussed the hydrogen ion only acts as a controlling factor on cell growth and activity when in abnormal concentration. It can now be stated therefore that before the factors affecting growth can be studied, the tissue must be planted in a medium whose physiological constitution is at least consistent with the survival of the cells. Strict attention must always be paid to salt concentration, osmotic pressure, hydrogen-ion concentration and the like before comparable results can be obtained as to the growth-promoting capacity of any other substances added to the medium. Slight deviations are not significant, and experiments up to now show that if cultures start in a physically correct medium any alterations that make it appreciably less physically correct will always in the long run prove to be noxious rather than beneficial.

Tissues then, explanted into a Ringer solution, and left undisturbed will survive until the medium becomes unsuitable or until their energy supplies are exhausted. There is evidence that the second is the more important limitation, for in such a solution no food substances are present, and the tissues are living on their own reserves, and it is not surprising therefore to find that the addition of glucose to the medium determines a greater area of growth and a more prolonged life (90, 91, 126). Krontowski and Bronstein (80) have, by microchemical methods, clearly demonstrated the disappearance of sugar from the culture media of actively growing tissues. In this connection it is particularly interesting to note that Watchorn and

Holmes [125] find that glucose is utilised in preference to protein, even when the latter is present in considerable quantities, thus demonstrating on the actual tissues the well-known protein sparing action of carbohydrates. Their experiments [73] are also interesting from other points of view. They cultured tissues in flasks in a medium of embryo extract, some floating freely, and others supported on cotton wool threads. Those that had no support showed no signs of active cell proliferation and migration and were found to produce only minute traces of ammonia and urea, while those that were supported and hence actively growing produced definitely measurable quantities of these substances. Tissues supplied with glucose in addition to the embryo extract no longer produced measurable amounts of ammonia or urea, thus indicating that the carbohydrate was used in preference to protein as an energy supply to the tissues. Besides illustrating the importance of glucose in the medium these experiments also emphasise the great difference in metabolism between actively growing tissues and those that are in a resting condition. This also will be referred to again in connection with somatic growth. The optimal concentration of glucose has not been determined. In hanging drop cultures (unchanged) the best results were obtained in 0·8 per cent. glucose [126]. These, although they started slowly, always grew well later and survived longest. In weaker concentrations growth was more active at first, but the supply was insufficient and the cells died earlier. Stronger concentrations tended to be inhibitory. Arguing from these results it would appear that a solution kept constantly at about 0·1 per cent. would be the most effective means of supplying glucose. Incidentally this is of course approximately the blood sugar level in most warm-blooded animals. Watchorn and Holmes found that in their flasks, where the supply of sugar was relatively inexhaustible, a concentration higher than 0·2 per cent. began to show signs of toxicity. They were using tissues from the kidney of the embryo rat, whereas previous work has mostly been done on chicken tissues.

This leads on to the question of oxygen usage by the tissues, on which the attentions of several investigators [68, 104, 123, 127] are at present centred, and the evidence is somewhat conflicting. In this respect the behaviour of the cells depends not only on the oxygen pressure, but also on the glucose available, and on the fate of the products of the glucose metabolism. If these are lactic acid and CO_2, as is generally supposed, they will influence the hydrogen-ion concentration of the tissue fluids. Therefore in interpreting the results of the various investigators, attention must be paid to their methods of culture.

Mottram [104] found that in the complete absence of oxygen no growth was obtained in a plasma medium. This he found was true both for normal and cancerous cells. Rat sarcoma cells he found would show activity in plasma at an oxygen tension of 20 mm. Hg, while normal tissues did not grow till the tension was raised to 80 mm. Short anaerobiosis did not kill the tissues but merely suspended animation. He also studied the effects of high CO_2 tensions and found that normal cells ceased activity at 250 mm. pressure, whereas sarcoma cells remained active till 300 mm. His conclusions therefore were that normal cells, as would be expected, need oxygen and are inhibited or killed by excess of CO_2. Sarcoma cells are more resistant.

Fischer[68], on the other hand, has grown cultures of various cell types at different depths of medium in test tubes, and finds that under these conditions the fibroblasts can show activity at greater depths (more severe anaerobiosis) than mouse carcinoma cells. Leucocytes resist these anaerobic conditions better than either of the other types. It is possible that mouse carcinoma and rat sarcoma may behave differently in respect to anaerobiosis, although according to Warburg[123] their capacities for anaerobic glycolysis are not materially different, both showing considerably more than normal tissues. Under the stagnant conditions in the tubes used by Fischer, therefore, the malignant cells would be expected to produce more lactic acid than normal tissues and consequently this would prevent growth sooner. Leucocytes grow scattered in the medium, and a possible explanation of their survival in anaerobic conditions is that the lethal concentration of lactic acid is not reached so quickly.

Wind[127] found that in the absence of oxygen and of sugar no growth was possible either for normal or for sarcomatous tissues, but that in the case of sarcoma cells if sugar was introduced a certain amount of activity could occur anaerobically. He thus illustrates the importance of glucose for sarcoma cells, and this may be compared with the work of Krontowski and Bronstein[80] who showed that the glucose consumption of carcinoma cells was higher than that of normal cells.

Warburg[123] has made considerable studies on the anaerobic glycolysis of which various tissues are capable, and finds that embryonic tissues are more potent in this respect than older tissues, while cancerous cells are more potent than either. He has also[124] been able to show the decrease of anaerobic glycolysis with age. Similar results were obtained by Burrows[14] who found that normal chick heart cells would survive and grow for a short time in pure nitrogen, but this capacity was confined to very young tissues, and for hearts from chicks of more than five days' incubation oxygen was necessary. Wind[127] has confirmed this.

The life of the tissues in simple saline media must however be short, even if glucose is liberally supplied, since the tissues have no nitrogen supply other than that obtained from their own substance, and the problem of supplying the tissues with nitrogen cannot yet be said to be solved. Embryo extract added to the medium causes increased activity, and Holmes and Watchorn[73] have shown that ammonia and urea result from this activity so that embryo extract presumably supplies the tissues with all the nitrogenous materials that they require, but in what form is still uncertain. Nor is it known whether embryo extract acts merely as a nitrogen supply, or whether it also contains growth stimulants. But before considering the properties of embryo extract, and other extracts which produce similar results, a discussion on the attempts to supply the cells with nitrogen will somewhat clear the pitch.

It has been assumed now for many years that the tissues of the body obtain their nitrogen from amino-acids, and consequently attempts were very early made to supply tissue cultures with these substances. But in the earlier experiments[13] sufficient attention was not paid to matters of concentration, and consequently the first investigators found them to be toxic. Ebeling[52] found that in lower concentrations they were not toxic, but produced no very beneficial results. Baker and

20-2

Carrel[2] worked with embryo extract and by dialysis considerably reduced its activity. The dialysate, however, temporarily activated growth, but did not allow the continuous survival which the whole extract produced. They attributed the initial activity of the dialysate to the fact that the amino-acids had passed through the membrane, and in confirmation of this they found that mixtures of amino-acids in low concentration produced similar results. They state however that such solutions only increase the area of emigrated tissue without increasing its mass, but since Wright[128] has shown that the same dialysate produces very large numbers of mitoses in the outgrowing cells, and the cells do not appear to decrease in size, the results seem to be at variance with one another. Also it is interesting to notice that Carrel found that trypsin digests of embryonic tissue were always toxic, even when diluted. Partial activity was restored to the dialysed embryo extract by addition of the dialysable portions, and similar results were obtained by addition of mixtures of amino-acids. From these results, Baker and Carrel[2, 3] conclude that amino-acids are of great importance to the cells, although they are not sufficient to keep them alive indefinitely. The evidence, then, on the use of amino-acids by cells in culture is anything but convincing, and further investigation in this direction is urgently needed. If the amino-acids are used at all, tissue culture methods should be ideal for studying their physiological properties as a class, and also their relative merits, since it is unlikely that they would all be equally valuable.

As a result of their investigations Carrel and Baker concluded that the nitrogen supply of tissues, or the substance which allowed indefinite survival and growth, was to be found in the protein fraction of embryo extract. Proteins have however from early days of tissue culture proved ineffective as a medium. Gelatin, egg albumen, egg globulin, and many other protein bodies have been found relatively useless, at best only slightly prolonging survival[3, 110, 119]. So they sought for the active constituent among the breakdown products of proteins and quickly discovered that after brief digestion of embryo tissues with pepsin they obtained a medium of great growth-promoting activity[39]. Prolonged digestion destroyed this activity, so that they concluded that the active body was among the higher cleavage products, *i.e.* not among the peptones. They therefore turned their attention to other proteins and obtained confirmatory evidence from digestion of fibrin and crystalline egg albumen. From the former and from Wittë's peptone which contains a relatively large quantity of higher cleavage products, they have prepared media which allow of good growth and indefinite survival. Having previously shown that acid metaprotein obtained from crystalline egg albumen is inactive[3], they regard the proteoses as being the active bodies, and presumably the main nitrogen supply to the tissues. This they have confirmed by preparing the proteose fraction from Wittë's peptone in a purer condition, and still find it active. Similar results have been obtained by Fischer and Demuth[67 a]. Digestion by pepsin of crystalline egg albumen produced a solution which, when added to the culture medium, enhanced growth but did not allow the tissues to live indefinitely, and in a more recent paper[5] Baker and Carrel have shown that additions of glycocoll and thymus nucleic acid improve matters, but do not put them right. Incidentally, in a previous paper[3], both sodium nucleate

and thymus nucleic acid were reported as being entirely inactive when added to the culture medium. As the result of some experiments not yet published, working in saline media, the author has so far been unable to confirm these results with Wittë's peptone and digests of fibrin, and has found the proteoses relatively inert. But if they should prove to be active, it will be a matter of great interest not only from the point of view of tissue culture, but also with regard to the normal methods of nutrition of the tissues in the body. The evidence, then, so far brought forward by the work of Baker, Carrel and others seems to indicate that tissues can obtain some energy from amino-acids, but that their nitrogen supply is chiefly obtained from proteoses: embryo extract contains both elements.

Apart from this there is no evidence as to the source of nitrogen which is most convenient and accessible to cells in culture; but there is now a considerable body of facts concerning substances which stimulate cells in culture to greater activity. For example, the behaviour of embryo extract itself suggests something more complicated than merely a collection of proteoses and amino-acids. Both these groups of substances are relatively thermostable, yet numerous workers with the exception of Heaton (71), who states that an activator for fibroblasts is still present in heated embryo extract, have found that embryo extract loses all virtue when heated, even only to 56° C. In this process some of the proteins will be coagulated, but it is almost inconceivable that they should bring down with them all the proteoses and amino-acids. It suggests rather that some necessity for cell life is destroyed or else adsorbed on to the protein precipitate. The latter alternative receives some support from the fact that passage through filter candles removes, or considerably reduces, the activating substance (23). Similarly, prolonged shaking renders the extract worthless. These facts suggest that the protein fraction may be of considerable importance, but all attempts to isolate an active fraction by precipitation and similar methods have been more or less unsuccessful, except to indicate that it may be associated with the globulins present in the juice (61). It seems probable that the really active substance may be something which is readily adsorbed, and so put out of action when the proteins are coagulated, or when the embryo juice is filtered. The active dialysate obtained by Wright (128) suggests that a very active constituent with a relatively small molecule has passed through the collodion membrane. This may be the actual stimulant which allows the proteoses, etc., in the embryo juice to be utilised by the tissues, so allowing the cells to survive indefinitely.

The fact that tissue cultures of very early chicken embryos are not successful, and the fact that observers are agreed upon the necessity of keeping the cells in numbers for successful cultivation, led Wright (129) to investigate how it is that in nature the early stages of the chick are ever successfully passed through. Yolk itself proves inactive when added to tissue cultures, but he found that on dialysing the yolk a substance passed through the membrane which behaved very similarly to the dialysate from embryo extract. He records cultures showing enormous numbers of mitoses, so that the idea of a specific stimulant to division seems almost necessary. However, he gives no data as to the size of the cells, which would presumably get smaller if division went on more rapidly than growth. The dialysate, like that from

embryo extract, gave a negative biuret reaction. In his account of this substance he draws attention to its use in the nutrition of the embryo chick. These experiments raise the question, which still remains unanswered, as to whether it is possible by suitably altering the medium to stimulate the tissues now to multiply by mitosis, now to migrate, or again to differentiate and function. All the evidence that so far exists points to the conclusion that function is antagonistic to growth, and the medium, using the term in its widest sense, is responsible for cell behaviour in this respect; but at present little can be said with regard to the effect of the medium on the relation between cell wandering and cell division.

The effects of extracts of several adult tissues on the growth of cells *in vitro* have been studied with again many conflicting statements as to the results. Nearly all extracts seem to bring benefit to the tissues, but some more than others [122]. Liver extracts appear always to have proved inhibitory, although Heaton [71] suggests that they also contain an activator, whose influence is generally masked. Spleen, Rous sarcoma, bone marrow, and leucocytes have all been described as yielding saline extracts which increase cellular activity [23, 61]. Heaton records bone marrow as being the most potent in this respect, other observers have found spleen most active. As mentioned earlier in this paper, leucocytes play an important part in the nutrition of cells. They can grow and survive indefinitely in serum or plasma [35], thus indicating that they are capable of obtaining all their necessary food substances from such a medium. In this respect they differ widely from such tissues as fibroblasts. Not only are they able to obtain food from serum for their own use, but they are able to hand it on to such cells as fibroblasts, which under the influence of leucocytic secretions, or as called by Carrel trephones, are then able to proliferate actively in serum. Degenerating cultures of fibroblasts can be revived by the addition of leucocytes, or their secretions, and Carrel [35] describes an interesting experiment in which cultures of leucocytes and fibroblasts were planted in plasma in the same vessel. The leucocytes grew well, but the fibroblasts soon languished, until the outgrowing leucocytes met the edge of the fibroblasts, when a quick revival took place. It is interesting to notice that both spleen and bone marrow are essentially lymphoid organs and probably owe their activity to this. Rous sarcoma cells also behave in culture in a manner similar to leucocytes. They tend to liquefy plasma and if muscle tissue is provided in the medium, do not require embryo juice. Probably all those tissues which activate growth *in vitro*, activate according to the concentrations of trephones which they contain; embryo extract is presumably rich in them, but what they will finally prove to be, whether proteoses or some other substance, is still an open question. From the more recent work of Baker and Carrel [5] there are indications that certain proteoses are more effective than others, probably according to their amino-acid content, though the question of the presence of slight traces of active impurities does not yet seem to have been definitely ruled out.

It is a well-established fact that embryo tissues grow very much more readily than adult tissues, and that the activity of tissues growing in plasma is inversely proportional to the age of the animal from which the plasma is obtained. Carrel and his school [1, 4, 29, 30, 31], working with serum, have shown that it becomes inhibitory if

present in too great a concentration in the culture medium, and have demonstrated that in serum there exist substances which activate the growth of fibroblasts, and also substances which inhibit it. The active substances are precipitated by CO_2 and it has been suggested that they are related to the globulins. The residual serum, deprived of the CO_2 precipitate, is more inhibitory than normal serum, and its inhibitory powers have been correlated with the lipoid constituents. Another inhibitory factor of less potency is believed to be present in the protein fraction. Serum from old animals contains an increased quantity of lipoids and a higher lecithin-cholesterol ratio. As is well known, for most cells function and growth (cell multiplication) are mutually antagonistic, not only in the body but also *in vitro*. In connection with this it is very interesting to find that lipoids prevent growth, for in experiments in perfusing hearts excessive perfusion leads to an inhibition of activity unless the lipoids washed out are restored to the heart. Possibly the conditions are analogous. The actual changes which occur in the plasma rendering it more inhibitory with age have yet to be more fully elucidated. Whether the action is due to loss of stimulant or to an increase in the amount of inhibitors such as the lipoids is still uncertain, but the evidence so far favours the latter view.

The question of the changes in the *p*H of tissue fluids with age may prove significant. As previously mentioned, Mendeleeff (103) found that in the guinea-pig the embryo *in utero* had blood which was very much more acid than that of the adult. It is doubtful, however, whether this can be correlated in any way with growth, since shortly after birth its blood *p*H has risen to 7·0, whereas for some weeks more growth will proceed at a very great rate.

In the tissues of early embryos there is a large quantity of growing tissue tightly packed and with a relatively small blood supply, and this, connected with the fact that for successful growth of cells in culture, the cells must be present in large numbers, and the medium stagnant, suggests that the cells forming metazoan tissues are dependent greatly upon one another. Fischer (64) has suggested that this dependence is due to the slow diffusion of metabolites or secretions from one cell to another. These diffusing substances he terms "desmones" and assumes that they travel by way of protoplasmic bridges. They are probably produced by all healthy cells, and are independent of the trephones described by Carrel. This is shown by the fact that fibroblasts sometimes languish and become unhealthy even in the presence of a sufficiency of trephones, and can be restored to activity again by the addition of healthy cells. An ingenious theory has been put forward by Burrows (15, 16, 17, 18) also based on the behaviour of cells in culture and their demand for company before growth will occur. He suggests that, in the presence of oxygen, the cells secrete a substance "archusia," whose function resembles that of the desmones of Fischer, except that it depends upon the concentration in which it is present. In strong concentrations, it seems to act like an enzyme, bringing about digestion and autolysis of the tissues; in slightly weaker concentration it allows the cells to digest fats and proteins, thus permitting them to grow readily in suitable media. In still weaker concentrations growth of the cells ceases, but they are still capable of functioning, while, if even more dilute, the cells are rendered incapable of

carrying on their ordinary activities and round off, so becoming dormant. The substance is water soluble and secreted by the cells. Hence it tends to accumulate under stagnant conditions, where growth is best observed, and also it tends to be washed away easily. This might explain why cells round off and become inactive when washed too thoroughly, and why they will not function or grow when isolated. The properties of this substance correspond somewhat to those of bios, and also to those of vitamin B, but at the moment the theory is merely in the hypothetical stage, and further evidence is urgently needed. Considerations as to the permeability of the cell wall, and the ease with which many of the water-soluble constituents can be washed out from tissues, immediately suggest reasons why the cells should not grow where there is a large volume of cell-free fluid surrounding them, and cell crowding will obviously reduce the loss by diffusion of these necessary substances. The fact that oxidations taking place in tissues are reduced even to zero by washing with water, bears upon this point, since one of the causes is believed to be diffusion of certain active soluble substances into the surrounding fluid. Succinic acid, and glutathione among others, have been mentioned in this respect (54). Such diffusion may also easily take place in saline solutions, although probably less readily there since the cell wall is left intact as a restraining influence. The injurious effects of treatment with excessive saline fluids is seen in experiments in perfusing the heart of the frog, where it is well known that venous perfusion, in which the Ringer solution passes directly through the heart, can only be continued for a short time, whereas aortic perfusion may be much more prolonged, since by this method a small volume of fluid goes backwards and forwards into the heart, and if properly oxygenated will maintain the heart in an active state for many hours. The heart brought to rest by excessive venous perfusion can be restored to normal rhythmical activity by the addition of tissue extracts. Such considerations then may throw light on why the normal isolated cell will not survive. On the other hand, sarcoma cells can be isolated or scattered in a medium and nevertheless will grow (61).

Heaton (71) has done considerable work on the relation of vitamin B to growth of cells *in vitro*, and finds that a watery extract of yeast has a dual action. It stimulates the growth of epithelium, but tends to inhibit the growth of fibroblasts, and this inhibition of fibroblasts is not overcome by the addition of embryo extract. He has shown that the accelerator for epithelium is different from the fibroblast inhibitor, as among other things they have different alcohol solubilities, and experiments using very potent preparations of the anti-neuritic vitamin lead him to the conclusion that the growth stimulator for epithelium is of this nature. It is thermostable and readily soluble in alcohol. It has no action on fibroblasts. His technique differs slightly from the one adopted by the author in some experiments on the influence of glucose on growth, and this may explain the fact that he readily obtained growth of epithelium from the intestine of the chick in purely saline media, whereas the author found that epithelium would only grow when glucose was present (126). The growth was then proportional to the concentration of this substance, or in other words glucose acted as a stimulant to epithelial growth. In this same paper Heaton calls attention to the interesting fact that the liver grows chiefly as epithelium up to the

eleventh day of incubation, and after that behaves like fibroblastic tissue. Needham (106) has found that at about this time there is a marked change in the cholesterol content of the liver.

The action of other vitamins on cells in culture has not been greatly investigated. Burrows (17) suggests that vitamin A is required for the functioning of cells, and is formed when cells are digesting proteins and fats and growing under the influence of a strong concentration of archusia (vitamin B?). He also indicates that vitamins A and B are antagonistic; and in functioning cells there is a balance between them. Most observers are agreed that fats and lipoids are inhibitory to the growth activities of cells, and as already remarked, Baker and Carrel (4) correlate the age changes in serum which retard growth with the increase in its lipoid content. This might suggest that age changes and differentiation of tissue are brought about by an upset of the balance towards vitamin A.

The experiments of Strangeways and Fell (116, 117) throw very interesting light on the behaviour of cells in culture, and also illustrate the value of the method from the point of view of studying the mechanism of differentiation in developing organisms. They have made use of the fact that under certain conditions of tissue culture uncontrolled growth gives place to somatic growth (120). That is to say that tissues instead of entering on a period of proliferation when cultured, carry on their development much more as they would if left in the organism. Some time ago Fischer (58) cultured small pieces of chick intestine embedded in tubes containing media of varying consistency, and found that they soon rounded off with the epithelial lining growing completely round them, so that they resembled small complete organisms, and the parts in the interior obtained their food substances through the epithelium. The type of growth depended on the type of medium. If this was fluid the stroma disappeared from the centre, so that the organism became hollow and cyst-like, and the epithelial walls showed well-developed villi. In more solid media the behaviour was even more interesting. The cells remained active and functional for long periods. That is to say muscular contractions were visible after a month's cultivation, and the epithelial cells continued to pour forth a mucous secretion, which apparently also liquefied the plasma, and after 48 hours' growth in a fresh medium the organism was always floating in a little lake.

Strangeways and Fell followed up this work by culturing the limb buds of chick embryos of 72–80 hours' incubation. They found that their behaviour varied according to the type of medium; and this had particular reference to its fluidity. If the cultures sank in the medium they became cystic in a few days, and these cysts often developed to a considerable size. In more solid media (plasma and embryo extract with or without saline) a period of uncontrolled growth occurred first, but this quickly ceased, generally leaving the culture surrounded by a layer of connective tissue or epithelium. The cultures were provided with a fresh medium every 48 hours, and specimens were fixed, sectioned, and examined each day. The cystic explants showed little of interest, but those growing in solid media continued to develop in a way similar to the course they would have followed if they had been left in the organism. Mitoses occurred freely and a process of differentiation went on. Cartilage, white

fibrous tissue, and typical epidermis all differentiated, but bone and muscle did not make their appearance. The general morphology of the cultures bore close resemblance to the normal limb skeleton, and this is in striking contrast with the result of control experiments, in which similar limb buds were grafted under the skin on the wings of young chickens. In these, differentiation went a little further, in that bone made its appearance, but the general structure of the graft bore no resemblance to the limb skeleton. This however was probably due to the abnormal stresses and strains to which the new tissue would be subjected.

After these successful experiments, they cultured (117) the developing eyes of chick embryos, and obtained similar but even more striking results. The eye was generally distorted beyond all recognition as an eye, but on microscopic examination clearly showed its capacity for self-differentiation. The pars optica of the retina, rods and cones, inner and outer nuclear layers, inner and outer plexiform layers, the pars ciliaris of the retina and the lens all developed during cultivation. That is to say that, except in morphological arrangement, which is probably normally dependent on the concomitant growth of the skeleton, the eye followed its normal course of development when completely isolated from the organism. In the later stages of culture the differentiation went on at practically the normal rate, but growth was retarded so that the cultures remained small, thus illustrating that growth and differentiation are essentially different processes and dependent on different conditions.

This type of work would seem to open up a tremendous new field for the study of the growth and development of early embryos. For some time now the eye has received considerable attention from experimental embryologists, and in certain amphibia the lens has been shown to be dependent for its development on the near approach of the optic cup (109). Once the epithelium has been influenced by the optic cup it becomes a self-differentiating unit, and then is only capable of developing into a lens. Cultivation of this epithelium *in vitro* before and after the approach of the optic cup might lead to interesting results. If extracts from the optic cup were made and their influence tested on the behaviour of the lens *in vitro*, a positive result would give striking confirmation to Spemann's theory of differentiation on the lines of specific chemical organisers (111). At any rate this self-differentiation of the eye *in vitro* is a result of exceptional interest and in the future tissue culture should help considerably in elucidating the problems of development.

There is one point of importance in connection with this work on differentiation and functioning of cells *in vitro* which arises partly out of the technique employed. This is the question as to what are the factors which determine whether the tissues shall migrate freely into the medium and show uncontrolled growth, or whether they shall remain externally quiescent and yet internally show considerable activity. Cultures grown embedded in the medium in tubes, after two or three sub-cultures, generally cease to proliferate externally and are found to have become enclosed in a continuous coating of epithelium or connective tissue. If the medium is solid these show differentiation and growth within, if liquid, they become cyst-like. The addition of foreign bodies near the growing culture, such as cotton threads, or as in

the hanging drop method the coverglass, favours uncontrolled growth. With chick tissues, therefore, the method of cultivation seems to be one deciding factor as to whether growth will be controlled or uncontrolled. Thomson (120) puts forward the idea that injured surfaces tend to show uncontrolled growth until surrounded by a new membrane or cells, and that an uninjured part tends to grow as a whole. This he accounts for in two ways, either it depends on the fact that the basement membrane is uninjured, or else the cells are subject to some central somatic control. The experiments of Murray (105) on Planarian tissues throw some light on the question, since she found that uncontrolled growth was comparatively rarely obtained from these tissues, which would always round off and tend to regenerate complete new worms. Planarian tissues have not been so specialised by differentiation as the tissues of the chick, and consequently can reorganise more easily, and are less dependent on their immediate surroundings. Chick tissues, on the other hand, depend on their position and immediate neighbours for their activity. If the organisation of the tissue is destroyed, as at cut surfaces where the membranes are injured, the cells tend to proliferate freely into the surrounding medium. If, on the contrary, the relationships of one cell type to another are undisturbed as in the experiments on growing eyes and limb-buds the cells continue to behave as they would in the body. Chick cells depend on one another for differentiation, and once differentiated they remain true to type, the process being irreversible. This is exemplified by the influence of fibroblasts and unfavourable conditions of the medium on the histological differentiation of epithelium which had previously shown uncontrolled growth. Planarian tissues, however, are to a greater extent totipotent. The process of differentiation is not so irrevocable, and their capacities for reorganisation are higher. But in both cases the type of growth obtained depends very largely on the conditions of cultivation, and the physical and mechanical make-up of the medium. The experiments of Holmes and Watchorn (73) suggest a marked difference in metabolism between actively proliferating cultures and those that are externally quiescent.

Harrison's original work on the outgrowth of nerves from the central nervous system of the frog definitely showed the way in which nerves develop, and many interesting experiments have been performed by Ingebrigsten (74, 75, 76) on the outgrowth of axis cylinders, and the degeneration and regeneration of nerve processes. From the point of view of developmental mechanics the experiments of Ingvar (77) are important. By using very weak currents, comparable in intensity to the electrical changes which are known to take place in the tissues of animals, he obtained a polarisation effect on the outgrowing cells of cultures. That is to say, if the current was passed directly through the medium the outgrowths of cells followed the direction of the lines of force, either from anode to cathode or vice versa, and showed certain differences according as they grew with or against the current. If the current was led by a single conductor across the medium the outgrowths all tended to be perpendicular to the conductor, again following the lines of force. If these experiments are confirmed they are of exceptional interest in indicating the possible nature of the directional forces at work on developing nerves during their outgrowth towards the organs they are destined to supply.

It is hoped that the brief account which has been given above will serve to indicate the varied nature of the subjects which have been already investigated by the method of tissue culture and the immense field for research in the future. Many of the results so far obtained are to some extent contradictory, as is perhaps to be expected in so young a branch of science, and this only serves to indicate the extreme importance of establishing standard methods. So many unknown factors enter into the behaviour of living cells, that the interpretation of results is made exceedingly difficult.

It has been impossible to treat all branches of the work in this review, and the account given of certain aspects is felt to be far from adequate, but the aim has been to indicate the importance and scope of tissue culture as a physiological method, and not to give detailed accounts of any particular branches.

Several books have recently appeared dealing with tissue culture (61, 66, 113, 114). Very extensive bibliographies are to be found in the books by Fischer (61, 66) and also the review articles by Carrel (37) and Carleton (20). W. H. and M. R. Lewis have also published an excellent account of cell behaviour in tissue cultures in *General Cytology*, edited by E. V. Cowdry. This contains a full bibliography. It has therefore not been felt necessary to compile a list of all papers referring to tissue culture from its physiological aspect, but to confine the present bibliography to those papers to which direct reference is made in the text.

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ANAEROBIC LIFE IN ANIMALS

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CONTENTS.

I. INTRODUCTION.

AT the end of the eighteenth century the new school of chemical thought headed by Lavoisier extended the recently developed theories of oxidation to the process of respiration in the living animal, and pointed out the essential similarity between combustion and animal metabolism. The early exponents of the chemical theory of respiration appear to have realised that this mechanism was necessary to supply the heat of the animal body, and to have given to the oxidation no other significance. As the theory became more firmly established, there grew up around the oxidation itself a conception of peculiar virtue for the maintenance of life, whilst at the same time the simple idea of an energy-producing process gradually receded. These changes in thought slowly excluded any scheme of energy liberation in the living organism, other than a simple burning of organic material to carbon dioxide and water.

So firmly rooted did the conception of life as a manifestation of oxidation become, that when Pasteur advanced his explanation of fermentation as "life without air" a storm of protest was aroused. It is not the function of the present review to follow in detail the controversy which resulted, but it is necessary, in order to understand the reasoning of the early workers on anaerobiosis in animals, to discuss some of the arguments used by Pasteur and the supporters of his views.

Pasteur recognised that living tissue required for its continuation a supply of energy, and that it was possible for the energy to be furnished by reactions other than direct oxidation with molecular oxygen. Apart from the bacteriological evidence from his own work, Pasteur quoted the original investigations of Spallanzani[64] in support of the view that life was not necessarily dependent on oxygen. The

work of Spallanzani was carried out almost simultaneously with that of Lavoisier, and was partly inspired by the discovery of oxygen. It consisted of the examination of the effect of inert gases upon animals, and resulted in the discovery of the power of organisms so widely different as animalculae and snails to live without the immediate presence of oxygen. During the ascendency of the oxidation theory this work had been entirely neglected, and its quotation by Pasteur undoubtedly suggested the possibility of processes, similar to those observed in bacteria, supplying the vital energy in larger and more highly differentiated animals.

In carrying through the controversy upon fermentation, Pasteur was forced to attempt to placate the opponents of his views by introducing what appears to be an unnecessary complication, in the form of the conception of intramolecular oxidation. His original assumption, that yeast fed on sugar, breaking it down not to carbon dioxide and water by oxidation, but to carbon dioxide and alcohol by means of a reaction in which oxygen was not involved, covered the facts without introducing any extraneous theories. It is perfectly obvious that the breakdown of sugar under these conditions must involve in some way the addition of oxygen to one part of the molecule at the expense of the other, but it is an entirely different matter to deduce the replacement of the normal oxidation process by such a mechanism in every case of anaerobiosis. How far Pasteur meant to carry this theory it is impossible to say, because it was seized upon and widely expanded by other workers, who were not so cautious, and who had not so clear a conception of its purely explanatory character as he had.

Pfeffer and Pflüger expanded the theory of intramolecular oxidation to all tissues living under aerobic or anaerobic conditions. It was assumed that the first step in the breakdown of carbohydrates was always a rearrangement within the molecule, by means of which carbon dioxide and alcohol were formed; to be followed immediately under aerobic conditions by the oxidation of the alcohol to more carbon dioxide and water. There does not appear to have been any serious evidence in support of this scheme, other than the occasional presence of alcohol in animal tissues in minute amounts.

Numerous investigations into the source of the alcohol gave varying results. Thus it was claimed that the presence of bacteria in the tissues would entirely explain the small quantities found. Harden and Maclean[18] lent support to this view by showing the possibility of the contamination of the preparation from the gut of the animal, and that such contamination leads to the formation of alcohol. There is little doubt that the early evidence of the presence of alcohol was obtained in this way. More recently A. E. Taylor[65] has finally proved alcohol to be a normal constituent of vertebrate tissues under the most aseptic conditions, but he has at the same time traced its origin to the reduction of small quantities of acetaldehyde. With this final explanation of the only experimental evidence in its support the theory of intramolecular oxidation was disproved, but in the meantime the possibility of a fermentation mechanism in certain animals had been the subject of long investigations.

Hermann utilised the theory of intramolecular oxidation in the same manner,

but he did not state his conclusions in terms of fermentation. He had established experimentally the power of isolated muscles to continue to contract in the absence of oxygen and after poisoning with cyanide. It would have been possible to suggest a fermentation process as the origin of the energy for the contraction, but Hermann did not do so, probably owing to lack of information as to the presence of any suitable end-products. The difficulty of the end-products could be avoided, however, if it were assumed that the carbohydrate in the muscle took up sufficient oxygen to oxidise itself completely, yielding large unstable molecules ready at any time to break down to carbon dioxide and water with the liberation of energy. These molecular complexes Hermann called "inogen," and he used them to explain in this way his observed phenomenon. This variation of Pasteur's original theory involves the absorption of the necessary oxygen along with the carbohydrate to form the giant molecules, but it otherwise only serves to extend the theory to more highly differentiated tissues. In one particular Hermann remained true to the idea of direct oxidation, he refused to apply his theory of the giant oxygen-containing molecule to the production of heat. Energy he maintained was produced in this way, but heat could only be produced by the direct burning of the tissue constituents.

These obsolete theories have been set out at some length, because they have influenced research into the nature of anaerobiosis in animals up to the last decade. The work of Bunge and of Weinland was inspired by them, and this work is still largely quoted in modern text-books, both on physiology and zoology.

II. EARLY VIEWS OF ANAEROBIOSIS AS A FERMENTATION PROCESS.

The distinction made by Hermann between the sources of heat and of mechanical energy in isolated muscle led to the first general investigation into anaerobiosis in animals. In 1883 Bunge(1), reasoning from this assumption, concluded that in the case of an animal living in a medium above its required body temperature there would be no need for heat production and hence for direct oxidation. The parasitic round worms of the mammalian intestine fulfil these conditions, in that they live in a medium maintained at the body temperature of the host. The other circumstances of environment are also favourable to a fermentation process, thus there is a large supply of food easily available and an almost complete absence of oxygen.

Bunge, therefore, set out to investigate the metabolism of the intestinal worm from the cat, *Ascaris mystax*, particularly examining the duration of life in hydrogen and nitrogen.

It is necessary in considering the earlier experiments in which animals were placed in an atmosphere of an inert gas, to bear always in mind the difficulties involved. There are two major problems, the preparation of the inert gas free from oxygen, and the displacement of the air in the experimental chamber by the purified gas. The method most generally used for the removal of oxygen from an inert gas is to bubble the mixture through alkaline pyrogallol, a method which under the most favourable conditions is not entirely effective. When, as frequently happens under experimental conditions, the stream of gas is rapid and the bubbles are big,

comparatively large quantities of oxygen may pass through the wash bottle. Thus a sample of nitrogen containing 1 per cent. of oxygen might quite possibly contain 0·5 per cent. after being treated in this way. Such a proportion of oxygen is sufficient to influence materially the duration of life in many of the invertebrates.

It is possible to pass a slow stream of an inert gas through a vessel, for periods much longer than would at first be expected, without removing all the oxygen of the air. The actual length of time depends on the shape of the experimental chamber, but unfortunately it is almost always such as to favour the formation of air pockets. This difficulty is easily illustrated in the case of the cockroach, which continues to move down to oxygen concentrations of about 1 per cent., but below that concentration behaves as if it were anaesthetised. The containing vessel used by the author in many experiments was a glass tube, of about 3 cm. diameter and 14 cm. in length, closed at both ends by rubber stoppers. If pure hydrogen were bubbled through this vessel at the rate of 20 c.c. per minute (*i.e.* about 60 bubbles per minute in an ordinary gas wash bottle) the cockroaches continued to move for 15–20 minutes, showing that not until after that time was the oxygen concentration less than 1 per cent.

The early workers do not appear to have taken any special precautions either to obtain oxygen-free gases or to remove completely the oxygen during the experiment. Hence the maximum duration of life as given in the older literature is almost invariably too great.

Bunge found that it was impossible to get satisfactory results when the intestinal worms were kept in a nutritive medium, owing to the bacterial activity. The whole of his results were therefore obtained from worms living without food in 1 per cent. sodium chloride solution. He found the worms to live for two weeks after the air above the saline had been replaced by hydrogen or nitrogen. For the reasons given above it is difficult to accept this result without reservation, but in a later type of experiment Bunge undoubtedly proved that anaerobic life was possible for periods up to five days. He placed a number of the animals in saline and then inverted the containing vessel over mercury. In this case there can be no doubt of the exclusion of all oxygen, except that dissolved in the saline. From a knowledge of the solubility of oxygen and the conditions of temperature and pressure at the beginning of the experiment, Bunge calculated that during the five days which the worms lived, they had available only 0·023 c.c. of oxygen per gm. of worm per day.

From this evidence it would appear that the worm *Ascaris mystax* can live for some days with an almost negligible oxygen supply. The power of continuous movement under these conditions is however less certain, as Bunge reports that during the experiment the animals became dormant and ceased to move except at long intervals. The gradual cessation of movement suggests the exhaustion of some material from which energy is being liberated anaerobically. This fact, considered in conjunction with great variation in the duration of life shown in experiments where oxygen was not rigidly excluded, suggests a condition of facultative rather than of complete anaerobiosis.

Bunge [2] followed his work on *Ascaris mystax* by the examination of a number

of other animals, whose method of life might possibly involve long periods without oxygen. Thus leeches and flat worms from the slime of ponds, and small arthropods which cannot breath continuously in their normal environment were selected. The actual results are given in Table I.

Table I.

Animal	Duration of anaerobic life
Leeches:	
Hirudo medicinalis	3 days
Hamopsis	2 days
Clepsine	6 days
Nephilis	2 days
Flat worms:	
(unspecified)	1–2 days
Lumbricus:	
(with haemoglobin)	2 days
Snails:	
(probably freshwater)	10–15 hours
Arthropods:	
Dytiscus, Asellus, Hydrachna, and small Crustacea	1–5 hours

It can only be concluded from these results, after making every allowance for the presence of small quantities of oxygen in the inert gases, that the ability to live, at least for a time, without performing any direct oxidation is relatively common amongst the invertebrates.

Bunge(3), having established the reality of this phenomenon of anaerobic life, attempted to show what reaction was responsible for the supply of energy under these conditions. He was prepared to find some process closely related in character to alcoholic fermentation, and proceeded to look for substances similar to the end-products of fermentation and bacterial activity in the medium in which intestinal worms had lived. He did not use for these experiments the relatively small worm from the cat, but those from the pig (*Ascaris lumbricoides*) and from the horse (*Ascaris megalocephala*). The technique was largely the same as in the early experiments, and the products of reaction identified were carbon dioxide and a strong smelling volatile fatty acid. A rough estimation of the carbon dioxide was made, but, owing to the difficulty in establishing the identity of the fatty acid, Bunge was forced to abandon the attempt without making any definite suggestion as to the type of the reaction involved.

The first theory of the nature of the chemical process from which the energy for the maintenance of anaerobic life is derived was put forward in 1901 by Weinland(68). He, like Bunge, worked upon the intestinal round worms from the horse and the pig. Weinland assumed the anaerobic mechanism to be the normal one in these animals, for it is known that in the intestine there can be very little free oxygen present at any time. From the commencement of the work therefore he differentiated the intestinal worms from the other cases examined by Bunge, in

which animals normally breathing oxygen had been temporarily forced to live without it.

Weinland adopted the same technique as that employed by Bunge in his early experiments, using 1 per cent. saline as the medium. His first experiments were designed to show the influence of the nature of the inert gas upon the duration of anaerobic life. As a basis of comparison he determined the length of life of the worms in saline with a plentiful supply of air, and found it to be about seven days. When the air was replaced by hydrogen or nitrogen, there was no change in the time, but with carbon dioxide it was increased to ten days. These results are open to the same criticisms as Bunge's work, so they cannot be accepted as quantitative observations.

It is particularly unfortunate that Weinland does not say by what criteria he judged the worms to be dead, as the dormant state already described may easily have misled him into under-estimating the period of life in some experiments.

Having satisfied himself that the worms were really anaerobic in habit, Weinland set out to examine the change in the body constituents during starvation, and the nature of the products of metabolism in the surrounding solution. A preliminary determination(67) of the glycogen content had already been made; this was now repeated with the addition of the reducing sugar (dextrose), the nitrogen (protein), the ether soluble matter (fat) and the water content. The only constituent which proved to be abnormal in quantity was the glycogen, which in some cases amounted to as much as 25 per cent. of the dry weight of the worms. The very high value for glycogen immediately pointed to this complex carbohydrate as one of the possible reactants.

When analyses were made after several days of starvation in saline, it was found that there was a marked decrease in the carbohydrate. The percentage loss was roughly the same for the glycogen and for the reducing sugar, the actual loss being of course chiefly at the expense of the large quantity of glycogen. The average value obtained for the carbohydrate loss over a number of experiments was 0·8 gm. per 100 gm. of worm per day.

The changes in the fat and protein being entirely secondary in character, Weinland assumed that carbohydrate was the material involved in the anaerobic production of energy.

The water content of the worms underwent a marked increase during the starvation period, a fact which Weinland did not satisfactorily explain. It seems probable, however, that the solution of 1 per cent. saline is hypotonic when compared to the fluids in the gut of the host, hence, if the body fluids of the worms are in equilibrium with the latter, water will tend to pass from the saline into the bodies of the animals. At the same time there will be a tendency for any dissolved material, to which the gut or the body wall of the worms is permeable, to pass out into the surrounding solution. The question of the transference of materials to the solution by diffusion is important in connection with the discussion of bacterial activity, and will be referred to again later.

In the surrounding solution Weinland found the same strong-smelling volatile

fatty acid which had been described by Bunge. This acid distilled in steam, and was slightly soluble in water. The smell was that usually associated with the lower members of the homologous series of saturated fatty acids.

· The steps taken by Weinland (68, 70) to identify this acid are open to considerable criticism. He distilled it from the aqueous solution, and continued to collect the distillate until sufficient water was present to form a homogeneous solution. From this solution he prepared a calcium salt, which crystallised on standing. The salt resembled those of butyric, valeric and caproic acids in general characteristics, and of butyric and valeric in being more soluble in cold than in hot water. In order to obtain further evidence the calcium in the salt was estimated as oxide by ignition, and an ultimate analysis was made. The percentage of calcium oxide varied in different experiments from 22 to 24; calcium valerate requires a value of 23·1, but mixtures of the calcium salts of the acids given above would give rise to values varying between 20·7 for calcium caproate and 26·2 for calcium butyrate. The ultimate analysis in the same way gave indefinite figures, which might be taken as indicative of the presence either of valeric acid or of a mixture of acids. Weinland held the view that the acid was valeric, and in an attempt to confirm this he recrystallised the calcium salt from water, and showed that there was no marked change in calcium content as determined by ignition. The separation of such a mixture of acids by crystallisation is a difficult one and it is very doubtful whether any change in the percentage of calcium oxide detectable by the methods used by Weinland could be produced except by long and careful fractional crystallisation.
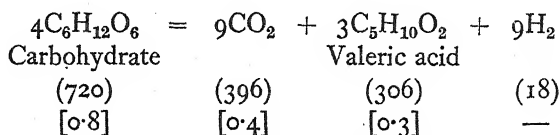
Weinland however regarded the evidence as sufficiently good to warrant the assumption that valeric acid alone is produced by the anaerobic metabolism of the worms. Having decided that he was dealing with a single acid, he made an attempt to determine the amount of the acid present in the fluid surrounding the worms. The solution was titrated with standard baryta, using phenolphthalein as an indicator, to obtain the total acidity; the precipitated barium carbonate was filtered off and estimated by ignition, and this value subtracted from the total acidity. The baryta used, other than by the carbonate, was calculated to valeric acid. Determined in this way the value for the production of valeric acid amounted to 0·3 gm. per 100 gm. of worm per day.

Weinland also measured the output of carbon dioxide, and found it to be greater under aerobic than under anaerobic conditions. Thus in the former case the yield per 100 gm. of worm per day was 0·54 gm. and in the latter only 0·38 gm. If the worms were truly anaerobic in habit such an increased output of carbon dioxide in air is not to be expected, but Weinland overcame the difficulty by suggesting that some additional activity is associated with the presence of air. Thus he suggested that egg production might be dependent on oxygen, or that the aerobic bacteria associated with the worms began to multiply. For purposes of calculation Weinland adopted the lower figure, rounding it off to 0·4 gm. per 100 gm. of worm per day.

On the evidence thus available Weinland put forward the theory of an animal fermentation process. The word fermentation in this case is undoubtedly used to indicate a process essentially similar to the production of alcohol by yeast or butyric

acid by bacteria, and hence in Weinland's view something entirely different from the normal metabolism of oxygen breathing animals. It is difficult, with our present knowledge of the multifarious enzyme actions occurring in the animal body, to grasp clearly the fact that the process here suggested was allying the intestinal worms with the micro-organisms as distinct from the higher animals.

The actual reaction involved in the production of energy Weinland wrote in the following equation:

$$4C_6H_{12}O_6 = 9CO_2 + 3C_5H_{10}O_2 + 9H_2$$

Carbohydrate   Valeric acid

(720)   (396)   (306)   (18)

[0·8]   [0·4]   [0·3]   —

Molecular weights thus ( ).
Grammes produced or consumed per 100 gm. of worms per day thus [ ].

This equation, apart from any criticism which might be advanced as to the nature of the experimental evidence involved, raised another difficulty by introducing a large amount of hydrogen, no trace of which could be found. Weinland attempted to overcome the objection, which he saw must inevitably follow, by postulating the presence of some substance capable of combining with the nascent hydrogen as it was produced. He does not seem to have realised that any such substance must be an oxidising agent, and also that a continuous supply must be available. It is difficult to understand how such a supply could be maintained without an ultimate use of molecular oxygen.

There is little doubt in view of the more recent evidence that Weinland was unwise to venture so complete a theory on the evidence he had obtained. It was however widely quoted without a critical examination of the experimental basis.

One serious criticism was however advanced, namely, that the whole of the facts could be explained on the assumption of bacterial activity. It was pointed out that in the intestine of the host are to be found large numbers of anaerobic bacteria, which produce mixtures of the lower fatty acids. Against this criticism Weinland pointed out the lack of food for the bacteria when the worms were kept in saline. Such a contention cannot however hold, for if the worms have the power to excrete valeric acid, it is equally reasonable to assume that they can put out other substances, into a hypotonic solution, which will act as substrate for the bacteria.

In order, if possible, to provide a conclusive proof that the fermentation reaction actually took place in the body of the worms, Weinland[69] prepared by the method of Buchner a pressed juice from *Ascaris lumbricoides*. This extract he attempted to sterilise by means of various antiseptics, and then allowed it to act upon dextrose. A reaction took place, the dextrose being broken down to carbon dioxide and the fatty acid identified by Weinland as valeric. If it were certain that the pressed juice was in fact sterile, this evidence would be unanswerable, but there was from the first some doubt, and more recent evidence has shown that it was justifiable.

The acceptance of the theory of an animal fermentation process for the intestinal worms proved a definite obstacle in the way of the development of a general theory of anaerobiosis in animals. Unless the actual observations were in error it did not seem possible to fit the case of *Ascaris* into the general scheme, which was being evolved for the other groups of animals. Although it necessitates a departure from the chronological development of the theme, it is simpler to consider at this stage a re-examination of the evidence in the case of *Ascaris*, in order that the logical sequence may be followed in the presentation of the later theories.

In 1924 Anton Fischer(5) undertook the repetition of some of Weinland's experiments. He allowed the worms to live in saline and determined the total acidity. A qualitative examination of the solution showed that apart from the fatty acid, described by previous workers, there was also present considerable quantities of lactic acid. Fischer estimated this acid and found that it accounted for 10 per cent. of the total acidity.

Instead of preparing a pressed juice Fischer mashed up the whole of the worms, and sterilised the pulp with toluene. On adding dextrose, or with the pulp alone, acid was formed. An examination of this acid showed it to contain no fatty acids, but to be made up entirely of lactic and phosphoric.

Unfortunately the work had to be given up at this stage owing to illness, but Fischer had definitely shown Weinland's evidence to be unreliable, and that any theory demanding the production of valeric acid as one of the end-products was untenable.

In the following year Slater(57) was able to furnish additional material in support of Fischer's work. He prepared a culture of the bacteria found with the intestinal worms in the gut of the host, and inoculated a nutritive medium containing dextrose. After incubation for 24 hours the solution smelled strongly of the lower fatty acids, and after further growth a mixture of acids similar to those isolated by Weinland could be distilled over.

Doubt having been thrown upon the validity of part of the previously accepted facts as to the metabolic processes involved in the intestinal round worms, the rest of the evidence was examined. The general conditions under which these worms live are such that, apart from movement, very little energy can be required. Hence it is of great importance to force them to keep in movement during the experiments on the duration of life in inert gases.

An arrangement was devised by means of which the worms could be stimulated at intervals, and three experiments were carried out side by side. In two vessels the worms were kept in hydrogen and in a third in air. Slowly the worms under anaerobic conditions ceased to respond to stimulus, whilst those in air moved freely. Air was next admitted to one of the vessels where the worms had ceased to move, and slowly movement was resumed. After a further 24 hours the worms without air were dead, whilst the other two batches were entirely normal.

From these experiments Slater concluded that the intestinal worms of this type could make use of oxygen when it was available, and further that under the conditions of the experiment, *i.e.* with a fixed amount of medium into which the

end-products could diffuse, they could not live anaerobically and perform muscular work for more than a limited time.

It is only necessary at this stage to show that the facts upon which the theory of animal fermentation were based are in themselves inaccurate, in order to proceed unhampered to the discussion of the development of the conception of hydrolysis as the source of anaerobic energy. The suggestion of an alternative theory is therefore left until a later stage.

III. THE DEVELOPMENT OF THE THEORY OF GLYCOLYSIS AS A SOURCE OF ANAEROBIC ENERGY.

In 1907 Pütter[53, 54, 55] confirmed the earlier observations of Bunge, that the leech could live for considerable periods without air. He, however, unlike Bunge, clearly differentiates between this case and that of the intestinal round worms. The metabolism of the latter he believed to be entirely anaerobic, whilst the former only substitute a special mechanism for normal oxidation process when the oxygen supply is deficient.

Pütter examined the liquid in which a number of leeches had lived with a limited supply of air, and found evidence of the presence of acetic acid and acetone. He already regarded these substances as intermediate products in the normal oxidation of carbohydrate to carbon dioxide and water, and therefore concluded that the difference between aerobic and anaerobic life was not a fundamental one. Adopting the general thesis that oxidation consisted of a number of steps, Pütter suggested that the early changes did not require oxygen, and hence they could proceed when air was removed, and liberate the necessary energy for maintenance of life.

The preliminary reaction in the oxidation of glycogen must consist of its hydrolysis to dextrose, and this Pütter took to be the energy-liberating mechanism. It followed that during anaerobiosis dextrose must accumulate in the tissues of the animal, and when a little air is available the partial decomposition products of the hexose will be found.

Pütter attempted to show that a considerable liberation of energy was involved in the glycolysis, by calculating from the heats of combustion of glycogen and dextrose, and, owing to an error, believed that he had done so.

When this error was pointed out by Lesser[29] and the very small amount of energy liberated by the glycolysis made clear, Pütter's explanation was discredited. It had however drawn attention to the possibility that the anaerobic mechanism was only a part of the normal chain of events, and not an entirely different process, and in this way has had a considerable influence on subsequent work.

The work of Lesser on the earthworm and the frog marks a transition stage in the development of a satisfactory theory of anaerobiosis. Although in the case of the earthworm he worked definitely on the basis of a special fermentation process, and in fact satisfied himself that such a process was responsible for the energy production in the absence of air, it is abundantly clear that he thought of the anaerobic metabolism only as an alternative to the aerobic. He rejected at first the

idea of glycolysis, and postulated two distinct reactions for the two forms of life, each in itself sufficient and entirely independent.

The earthworm was selected by Lesser (26, 28, 30, 31, 37) as the most suitable animal for examination. The determinations made were similar to those of Weinland, with the addition however of experiments on the aerobic metabolism for purposes of comparison.

A series of determinations were made of the glycogen content under various conditions. It was found that when the earthworm is allowed to starve it draws almost entirely on its glycogen reserves up to 20 days, hence in experiments on these animals it is not necessary to consider fat or protein metabolism. The falls in glycogen content in air and in nitrogen were compared, and it was found that the former was only about one-sixth of the latter. Carbon dioxide was given off under both conditions, in not markedly different quantities, and a volatile fatty acid was produced. This acid was similar to that claimed by Weinland to be valeric, but Lesser could not satisfy himself as to the identity of the product from the earthworms. There was also a marked difference between the amounts of acid produced in the presence and absence of oxygen. The normal output was small, but when the supply of air was cut off it increased to three times its previous level.

The results obtained by measuring the respiratory exchanges were not uniform in character, and are in consequence difficult to interpret. When the period following anaerobiosis is compared with the normal respiration, diametrically opposite results are obtained from different sets of measurements. In the one it appears that after anaerobiosis there is a rise in respiration with a fall in the respiratory quotient, and in the other little change in either the oxygen intake or the carbon dioxide output.

One other point observed by Lesser, although of little immediate importance in connection with his theory, is of considerable interest. When the earthworms have been for a time in an atmosphere of hydrogen they behave as if they were narcotised, thus indicating that either the accumulation of the products of metabolism or the continued lack of oxygen itself is affecting the nervous system.

Lesser concluded as the result of his experiments on the earthworm, that in this animal the oxidative mechanism and the fermentation process were taking place side by side under normal conditions, with the oxidation largely predominating. Such an assumption explained the formation of small quantities of the fatty acids when the worms were living in air. According to this theory, as the air was gradually removed the balance of activity passes over to the fermentation process.

The reaction involved in the anaerobic mechanism is according to Lesser similar but not identical with that in the case of the intestinal worms. It was impossible to establish quantitative relationships in the earthworm on the basis of the equation given by Weinland, owing to the fact that the carbon dioxide and the fatty acid only account for about half the glycogen disappearing. The increased amount of glycogen used is explained on the grounds of the low efficiency of the fermentation reaction, and Lesser contented himself by leaving the fate of half the carbohydrate explained by the formation of some unknown and unidentified material. The rise

in oxygen intake and lowering of the respiratory quotient after anaerobiosis, at first observed, he explained as due to the removal of the accumulated fatty acid by oxidation.

The acceptance of the idea of the piling up of end-products, during anaerobiosis, and their subsequent removal by oxidation marks a very definite advance in the theory of anaerobic metabolism. We see that in this Lesser and Pütter agreed, and from this time onwards the purely temporary character of the anaerobic process in animals may be regarded as accepted.

A still further advance was made by Lesser (27, 32) in his work on anaerobiosis in frogs. As early as 1875 Pflüger (52) had shown that frogs can continue to live at very low oxygen pressures. Lesser confirmed this fact, and carried out measurements of the heat output in air and in an inert gas. He found that the removal of oxygen caused the heat production to fall to half.

The drop in heat production precluded the possibility of the continuation of the normal oxidation process by means of stored oxygen, unless the metabolic rate were reduced to half in the inert gas. A measurement of the carbon dioxide output, Lesser argued, should answer this question. Unfortunately the output was found to be variable, amounting to the normal value in air during the first hour of anaerobiosis, and then decreasing during the rest of the time before the readmission of air.

Lesser was aware of the work of Fletcher on isolated muscles, and that in many ways this had given similar results to those he wished to explain in the case of the intact frog, but he hesitated to draw conclusions from this fact. He concluded therefore that the frog must also have an alternative fermentation mechanism.

Two years later Lesser published an account (33) of the determination of the glycogen content of frogs before a period of anaerobiosis, at the end of such a period, and after a further period in air. The quantity of glycogen fell rapidly during the time when oxygen was not available, but during recovery part of the glycogen reappeared.

Lesser, in order to prove the universal character of the disappearance of glycogen, as distinct from the special case of the muscles, examined the liver. The proportion of carbohydrate lost was the same for the liver as for the whole animal, and therefore he assumed that the anaerobic metabolism is the same for all the body tissues.

The explanation of these facts given by Lesser is a variation of the theory of Pütter. Thus he assumed that the glycogen is hydrolysed to dextrose in large quantities, to yield a part of the necessary energy. A portion of the dextrose then undergoes further decomposition to an unknown material to produce the rest of the energy. When air is again available the dextrose which has not undergone further decomposition is resynthesised to glycogen.

Lesser and Grode developed this theory of glycolysis further (36) by examining the nature of the enzyme action which was responsible for the hydrolysis of the glycogen. They found it to be similar to that taking part in the production of lactic acid from glycogen in injured muscle, as described by Fletcher and Hopkins (7). A careful examination of the blood and urine of the frog for the reducing sugar proved unsatisfactory.

Lesser had thus provided most of the necessary evidence for a theory of anaerobiosis based upon the lactic acid cycle, but he failed to connect the disappearance of the glycogen with the formation of lactic acid.

In 1916, A. V. Hill[21] suggested for the first time the possibility of explaining Lesser's observations of heat production and glycogen usage entirely on the basis of the production of lactic acid. If it had then been known that lactic acid is resynthesised to glycogen in isolated muscle, the essential correctness of the suggestion must have been obvious.

Lesser[34] took up the question afresh in 1923. He was then prepared to accept the presence of lactic acid, but only attributed part of the reaction to its formation. He determined the quotient Calories evolved /Gm. lactic acid formed and obtained for it the value 361, which is in close agreement with that observed by Meyerhof[43] in isolated muscle. He did not however consider the cases to be parallel owing to the difference in the ratio of the carbon dioxide output to the lactic acid formed, and from a consideration of the heat measurements concluded the formation of lactic acid to be accompanied by considerable hexose formation and some other reaction, so far unidentified.

The results were complicated in the frog by differences observed in animals at different times of the year, and the production under certain conditions of considerable quantities of reducing materials, an observation which supported the suggested hexose formation.

Further work by Lesser[35] on the changes in glycogen content of the liver and other tissues is difficult to interpret owing to the possible transference of hexose during the experiment, and hence can only be accepted with reservation. The conclusion to be drawn appears to be the absence of resynthesis of glycogen from the liver, but as the liver is probably breaking down glycogen to supply carbohydrate to the other body tissues, the fall in glycogen content does not necessarily prove no synthesis to have occurred in this organ.

IV. THE DEVELOPMENT OF THE THEORY OF LACTIC ACID FORMATION AS THE SOURCE OF ANAEROBIC ENERGY.

Fletcher and Hopkins in 1907[7] published an account of their work on the relationship between muscular activity and lactic acid formation. An isolated muscle was known to decrease in irritability in an inert gas, until finally it ceased to respond to stimulation. If however air were readmitted at this stage, the muscle became once more active. The lactic acid content of the muscle followed this change in irritability very closely, rising in the absence of air to a maximum corresponding to the passive condition of the muscle, and disappearing when oxygen was again available.

Meyerhof[45] was able to show that the maximum could be increased by allowing the lactic acid to diffuse away, and that it was not the total amount of acid formed but the concentration in the muscle which influenced the activity. The lactic acid formed also showed a definite relation to the isometric work[43], thus clearly

indicating that the acid production was a step in the chain of events culminating in the contraction of the muscle.

The source of the lactic acid was shown by the same worker (44) to be the glycogen of the muscle. Thus we get a picture of the anaerobic mechanism as the breakdown of glycogen to lactic acid; a process which can continue to liberate the necessary energy until the hydrogen-ion concentration of the tissues is seriously affected by the acid.

The amounts of lactic acid found in resting muscle were very small, and hence it was impossible to say on the evidence then available whether the breakdown of glycogen to lactic acid was the source of muscular energy under aerobic conditions. The myothermic measurements of Hill and his collaborators (22), however, established the essential similarity between the initial output of heat in an isometric contraction, whether it takes place in air or in an inert gas. In air there is a further gradual output of heat after the first rapid outburst. The same workers were further able to show, when a muscle which had been contracting anaerobically was returned to air, excess heat was liberated equivalent to the sum of the differences between the total heat in air and in nitrogen for each contraction.

From this work it became clear that lactic acid is produced anaerobically during muscular activity and then removed by an oxidative process. Under anaerobic conditions the lactic acid accumulates, and is only removed when oxygen is once more available. In its removal the same quantity of oxygen will be required, which would have been used if the oxidation process had followed immediately on the contraction. Thus we get the conception of an oxygen debt. A man running at a speed greater than that for which his heart and lungs can supply the necessary oxygen accumulates lactic acid in his tissues, using afterwards for its removal the oxygen which he could not obtain during activity. The limit to which lactic acid may be stored either in isolated muscles or in the whole organism, and hence to the oxygen debt, is fixed by the power of the tissues to buffer the lactic acid, and thus maintain the irritability of the muscles.

It had been observed that carbon dioxide was produced during anaerobic contraction, and this fact had been used in support of the older theories of intramolecular oxidation. Fletcher now explained this on the basis of lactic acid formation (6). He showed that the carbon dioxide was preformed in the muscle as bicarbonate, and was evolved only as part of the buffering mechanism.

There remained unexplained the fate of the lactic acid during the oxidation process. Parnas (50) advanced some evidence to show that sufficient oxygen was used during recovery for its complete oxidation, but the heat measurements of Hill (19, 20), and Peters (51), and later the more careful estimation of the oxygen intake by Meyerhof (42), established definitely that only about one-fifth of the lactic acid or its carbohydrate equivalent was oxidised.

The fate of the rest of the lactic acid was ultimately explained by Meyerhof (44), who showed that it was returned to the glycogen from which it had come. We thus have a cycle of events, in which the carbohydrate starting as glycogen breaks down to lactic acid anaerobically and is reformed into glycogen by an aerobic mechanism

at the expense of the combustion of one-fourth to one-fifth of the reacting material, *i.e.* either lactic acid or glycogen. During any period in which oxygen is not available, the amount of glycogen disappearing will be between three and four times as great as when oxygen is present, owing to the fact that no resynthesis takes place.

So far the only tissue considered in connection with the lactic acid cycle has been muscle, but there is evidence available to show that such a cycle is general throughout the various vertebrate tissues. Levene and Meyer [38, 39, 40, 41] have shown that sterile kidney tissue and leucocytes each have the power to convert dextrose into lactic acid, whilst Kraske [25] demonstrated the same property in whole blood. Embden [4] was also able to detect *d*-lactic acid when a liver rich in glycogen was perfused.

Warburg [66] has finally established the important part played by lactic acid in the metabolism of all tissues. By means of a manometric method he was able to measure simultaneously the oxygen intake of thin slices of living material, and the carbon dioxide driven off from a bicarbonate solution by the lactic acid produced. For almost all the normal organs of the vertebrate body which he tried, he established the general relationship that when the cells were deprived of oxygen lactic acid was formed in quantities roughly four to five times as great as that which the oxygen would have oxidised. In the same tissues no lactic acid accumulated when a plentiful supply of oxygen was available, but as soon as the oxidation began to fail lactic acid appeared.

There are a few normal organs, whose cells seem to have a faulty oxidation mechanism and which in consequence produce lactic acid in air, the most important of these are retina and testes, and embryonic growths. In the case of carcinoma tissue this condition is general, but we find when no oxygen supply is available there is a corresponding rise in lactic acid content to the loss of oxygen, just as in normal tissues.

Meyerhof, Lohmann and Meier [47] have advanced still further evidence in favour of the presence of the lactic acid cycle in muscle and liver strips living in the presence of air. They added lactic acid to the solution in which such strips were placed, and obtained a rise in oxygen intake, a disappearance of the lactic acid and the formation of glycogen. Moreover, the oxygen used was only about one-fifth of that required for the complete oxidation of the lactic acid lost, and the glycogen found amounted to about four-fifths of the amount required for a theoretical conversion.

It seems reasonable therefore to assume generally for isolated animal tissues, the source of energy in the absence of oxygen to be the breakdown of glycogen to lactic acid, followed by the subsequent removal of the lactic acid when oxygen is once more available.

Such an assumption places the study of anaerobiosis in the intact animal upon an entirely different footing. There ceases to be anything remarkable in the fact that animals live for a limited time without oxygen. The problem therefore resolves itself into a re-examination of the older evidence in the light of this theory, the accumulation of certain new evidence to show that the deductions made are fulfilled, and

finally the provision of an explanation of the rapid collapse of the higher animals when the oxygen supply is stopped.

If the energy for the maintenance of anaerobic life in the intact animal is supplied by the first phase of the lactic acid cycle, it should be possible to observe certain phenomena when animals are kept without air. These may be summarised as follows:
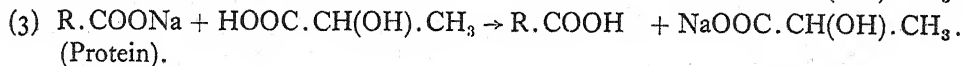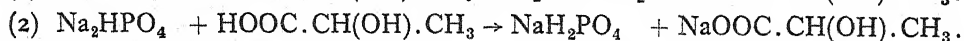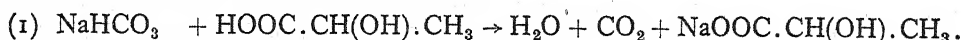
(1) When the oxygen supply is cut off, the glycogen used should increase by four to five times, and at the same time lactic acid should begin to accumulate.

(2) On readmitting air the lactic acid should disappear, and the glycogen be partly resynthesised.

(3) As a corollary to these deductions, the period of anaerobic life should be limited, and during recovery an amount of oxygen should be used in excess of the resting requirement by the amount lost during the anaerobic period (*i.e.* the amount used at rest in a similar period in air).

(4) The oxygen debt and the lactic acid content should be related, both as to time and to quantity, and in the same way the recovery of the oxygen debt and the lactic acid removal.

(5) Finally, carbon dioxide should be evolved during the period of oxygen lack and retained during recovery as a part of the buffering mechanism. This consists of three buffering reactions, all of which are more or less involved, and which may be represented generally by the equations:

(1) $NaHCO_3 + HOOC.CH(OH).CH_3 \rightarrow H_2O + CO_2 + NaOOC.CH(OH).CH_3.$

(2) $Na_2HPO_4 + HOOC.CH(OH).CH_3 \rightarrow NaH_2PO_4 + NaOOC.CH(OH).CH_3.$

(3) $R.COONa + HOOC.CH(OH).CH_3 \rightarrow R.COOH + NaOOC.CH(OH).CH_3.$
   (Protein).

Although the change from sodium hydrogen carbonate to carbonic acid involves the greatest increase in acidity for the neutralisation of a given quantity of lactic acid, it is still the most efficient means available, owing to the ability of the organism to rid itself altogether of the carbon dioxide. When in recovery lactic acid is removed and alkali is liberated, the equilibrium can again be restored by retaining a part of the carbon dioxide, which is being produced by oxidation in the tissue.

In the older work there is ample support for all these points, except those dealing directly with the production of lactic acid. Thus in the case of the intestinal worms Bunge and Weinland described a large diminution in glycogen content during anaerobiosis, and an output of carbon dioxide. No direct measurements were made on respiratory exchanges as the worms were believed to be entirely anaerobic, but from the difference in the duration of life between experiments in which the air was rigidly excluded and those less carefully performed, it would appear that oxygen can be used when it is available.

There remained however certain difficulties to explain; thus, if the worms are only temporary anaerobes, and require oxygen to remove the lactic acid formed, it is difficult to understand how they get a large enough supply in the gut of the host. Slater(57) has suggested that during the period of digestion the portal circulation

is sufficiently rich in oxygen to maintain a definite concentration at the gut wall, but this view is open to some criticism. The more probable explanation is furnished by Fischer's observation, that the fluid in which the worms have lived contains an appreciable amount of lactic acid. This suggests the possible leakage of lactic acid through the gut or body wall of the animal into the intestinal contents of the host, a process which, with the large food supply available, might yield the necessary energy without oxidation. It is not essential to preclude oxidation altogether, in fact the evidence seems to suggest the reverse; but oxygen would not be required, provided the surrounding fluids were continuously changing. Where the worms were kept in a closed space, the concentration of lactic acid would gradually rise until diffusion was stopped, as in the case of an isolated muscle in a limited volume of Ringer solution.

The other difficulty is to explain the production of fatty acids in saline. In order to attribute these substances to bacterial activity it is necessary to have foodstuff available. No satisfactory evidence has been advanced in explanation of the source of such a supply of material, and we are forced to fall back on the speculative suggestion previously mentioned, viz. that carbohydrate diffuses from the body of the worm into the hypotonic solution of saline.

In the case of the earthworm the experimental data is much fuller, and leads to less ambiguous conclusions. Lesser, as we have seen, not only showed that there was a marked fall in glycogen content during starvation, but also that this fall was very much greater during anaerobiosis than when air was present. The measurements on respiratory exchanges suggest for the first time, though not very clearly, an increased oxygen intake after anaerobiosis and a fall in respiratory quotient. The same author's subsequent work on the frog repeated the observations with regard to the glycogen, and added in addition the fact that part of the glycogen which had disappeared during the anaerobic period was reformed during recovery.

The formation of the volatile fatty acid by the earthworm was again a stumbling-block in the way of a simple explanation, and as before it can only be explained as being due to bacterial activity. There are in the intestinal tract of the earthworm bacteria capable of forming the lower fatty acids, but the source of supply of substances on which these bacteria can act is so far only a matter of speculation.

Thus with little exception the evidence available fits into the conception of lactic acid production as the source of anaerobic energy. The further examination of the theory by experiments particularly devised to test it is a comparatively simple matter, consisting of more careful manometric observations on the gaseous exchanges, and chemical determinations of the variations in the lactic acid content.

Davis and Slater have recently made these experiments, using as the animal for investigation the common cockroach (*Periplaneta orientalis*). This insect has numerous advantages for the work; thus, it does not normally move when left undisturbed, it behaves as if it were anaesthetised when it is deprived of oxygen, and it is available all the year round in large quantities.

The first evidence[60] sought after was the quantitative agreement between the oxygen debt—as measured by the normal oxygen used in air in a time equal to the

anaerobic period—and the oxygen used in excess over the resting requirements during recovery. Measurements were made of the oxygen intake in the same cockroach before and after a period of anaerobiosis. The first value supplied the normal resting usage, and the second the excess during recovery. The recovery in these experiments was followed until the resting value was again reached.

The results were in good agreement with the theory, varying only by such amounts as may be expected from the nature of the experiment. A number of results are summarised in Table II.

Table II.

Experiment	Duration of anaerobiosis (hours)	Calculated oxygen debt (mm. reading)	Excess oxygen (mm. reading)					
			½ hr.	1 hr.	1½ hr.	2 hr.	3 hr.	4 hr.
1	0·5	15	7	11	14	16	16	—
2	0·5	14	5	9	14	18	—	—
3	0·5	29	16	21	27	27	—	—
4	1·0	43	—	14	—	33	43	45
5	1·0	35	—	20	—	26	35	—
6	1·25	67	—	17	—	40	64	72

The modification of the Haldane blood gas apparatus, which had been used for these experiments, was not suitable for recording the simultaneous changes in carbon dioxide output, but by means of an adaptation of the Shakespeare katharometer (58) it was ultimately possible to make these measurements. The results (59) show a marked fall in the respiratory quotient during recovery (see Table III), with a gradual return to the normal value as the debt is repaid.

Table III.

Experiment	1	2	3	4	5	6	7
Normal respiratory quotient	1·00	0·98	0·83	0·93	0·70	0·93	0·89
Post-anaerobic respiratory quotient:							
Calculated[1]	1·00	0·98	0·86	0·95	0·73	0·94	0·891
Found:							
1st half hour	0·60	0·87	0·72	0·65	—	—	—
2nd half hour	0·83	0·91	0·68	0·63	0·62	0·82	0·53
3rd half hour	1·03	0·98	0·73	0·74	0·71	0·83	0·95
4th half hour	0·96	0·97	0·83	0·83	0·81	—	0·78

[1] The post-anaerobic respiratory quotient is calculated as follows. Suppose the normal oxygen intake is x cub. mm. per hour, with a respiratory quotient of 0·8, and the intake following anaerobiosis is $(x + y)$ cub. mm. per hour, then it may be assumed, that during recovery the basal metabolism continues, producing $0·8 \times x$ cub. mm. of carbon dioxide, and the additional y cub. mm. of oxygen are required to oxidise carbohydrate in the removal of lactic acid, yielding y cub. mm. of carbon dioxide. Thus the total carbon dioxide output to be expected will be $(0·8x + y)$, and the respiratory quotient $\left(\dfrac{0·8x + y}{x + y} \right)$.

The actual relationship between the oxygen debt and the carbon dioxide exchanges is an extremely complicated one, depending upon the liberation of lactic

and probably phosphoric acids, and their buffering by bicarbonates, phosphates, and protein. It is not possible therefore to look for a particular ratio between the oxygen debt and the carbon dioxide retained during recovery, but the figures obtained suggest a definite connection between these values. Table IV gives the oxygen debt and the carbon dioxide retention in the same experiments, and the ratio $\dfrac{\text{Oxygen debt}}{\text{Carbon dioxide retained}}$.

Table IV.

Experiment	Oxygen debt (cub. mm.)	CO_2 retained (cub. mm.)	Ratio $\dfrac{\text{Oxygen}}{\text{Carbon dioxide}}$
1	58	39	0·67
2	58	51	0·88
3	101	82	0·82
4	60	32	0·53
5	50	46	0·92
6	63	54	0·87

The most fundamental relationship, that of oxygen debt to lactic acid production was next investigated (61), but owing to difficulties in the chemical determinations due to the heavy cuticle and adventitious lactic acid in the gut, no clear correlation could for a time be observed. A careful repetition resulted in more reliable results from which it was possible to obtain the required information.

The lactic acid content slowly increased from the commencement of the anaerobic period; the rate of increase was dependent on the temperature. Until the concentration of lactic acid reached about 60 mg. per cent. on the whole insect, the relationship between duration of anaerobiosis and lactic acid content was almost linear, but beyond this value there was a slowing up of the acid formation, which became more and more marked. In order that the lactic acid should reach 90 to 100 mg. per cent., it was necessary that the anaerobiosis should be continued to a stage which was known to be fatal to a large number of the insects. The figures for the formation of lactic acid are given in Table V.

Table V.

Series	Temp. ° C.	Duration of anaerobiosis	Number of results	Lactic acid found (mg. %)	Excess over resting lactic acid (mg. %)
I	—	Control	4	18·8	—
II	14	1 hr.	2	37·9	19·1
III	14	2 hr.	4	55·0	36·2
IV	18	10 min.	2	27·5	8·7
V	18	1 hr.	5	57·2	38·4
VI	18	2 hr.	3	86·6	67·8
VII	25	1 hr.	3	72·6	53·8
VIII	25	2 hr.	3	98·8	80·0

The corresponding disappearance of the lactic acid during recovery after an-aerobiosis was shown by comparing the rate of oxygen recovery in one set of insects, with the rate of removal of lactic acid in other batches. The agreement in this case was also within the limits of experimental error. When however we consider the actual stoichiometric relationship of the lactic acid formed to the oxygen which would normally be used during a similar period, it is immediately obvious that some fundamental error is involved. If the calculation is made on the assumption that one part of lactic acid—or its carbohydrate equivalent—is oxidised for each 4·4 parts removed, the figure found by Furusawa and Hartree(9) for isolated vertebrate muscles, the ratio lactic acid found/lactic acid calculated is about one-fifth instead of unity. The constancy of this value however suggests a fundamental error affecting all the results alike.

The actual rate of formation of lactic acid is approximately the same as that in isolated amphibian muscles, and the maximum value consistent with activity is also similar in the two cases. On the other hand the oxygen intake in the cockroach is much higher, weight for weight, than in the animals in whose isolated tissues gly-cogen resynthesis occurs. From these facts all the lactic acid was assumed to be burnt, and the calculation was repeated on this basis. The figures in Table VI show a close agreement between the values found and those so calculated.

Table VI.

Series	Excess lactic acid found mg. %	Excess lactic acid calculated assuming		Ratio $\frac{\text{Found}}{\text{Calculated}}$	
		(1) resynthesis as vertebrate muscle mg. %	(2) complete oxidation mg. %	(1)	(2)
II	19·1	93·0	20·7	0·21	0·92
III	36·2	186·0	42·2	0·20	0·86
IV	8·7	23·0	5·2	0·37	1·67
V	38·4	138·0	31·3	0·28	1·23
VI	67·8	276·0	62·5	0·25	1·08
VII	53·8	270·0	61·6	0·20	0·88
VIII	80·0	540·0	122·0	0·15	0·66
			Average	0·24	1·04

The fundamental relationship between anaerobic life and lactic acid production may therefore be taken as established in the case of the cockroach; the way in which the lactic acid is removed by oxidation, although of great interest, is entirely secondary in the present connection.

The examination of an animal with a normal recovery mechanism was however desirable as a final confirmation of the general applicability of the hypothesis. The earthworm according to Lesser uses more glycogen under anaerobic conditions than when it has available a sufficient supply of oxygen, an observation which may be taken as indicative that glycogen resynthesis occurs.

Slater and Davis [62] have determined the respiratory exchanges and the lactic acid production for the earthworm. They found a good agreement between the calculated and measured oxygen debt, and between the calculated lactic acid content —on the basis of one part oxidised to 4·4 parts removed—and the value determined by chemical analysis. These results together with those of Lesser provide all the data necessary to show, for the earthworm, that the anaerobic metabolism of the whole animal is the same as that observed in isolated amphibian muscles.

Further investigation may in some way contradict the general theory of anaerobiosis here involved, but so far as the present evidence goes, the production of lactic acid from glycogen seems to be well founded and sufficient to explain all the available facts.

V. THE ENERGETICS OF ANAEROBIC METABOLISM.

Throughout the preceding discussion it has been repeatedly necessary to emphasise the energetics of vital metabolism, and to keep clearly in mind the energy available from any reaction considered.

In one particular all the reactions which have been suggested are in agreement, viz. the position of glycogen as the material decomposing. In no case is fat or protein assumed to enter directly into the process as a primary reactant.

Glycogen can only be decomposed by way of hydrolysis to dextrose or to some dextrose derivative. When however this preliminary glycolysis has occurred, the course of the reaction may vary in an almost unlimited way. It is therefore useless to consider the energy production in processes other than those for whose existence independent chemical evidence is available.

The earlier theories of anaerobiosis can be quickly dismissed. The fermentation process of Bunge and Weinland were ill-defined, and it is impossible to make any calculations on the evidence available. The theory of glycolysis suggested by Pütter and Lesser fails to explain the source of energy in sufficient amounts for the maintenance of life. Thus, taking the highest value for the heat of combustion of glycogen [56], viz. 3836 cal. per gm., in dilute solution, and subtracting the heat of combustion of dextrose in the same state [63], 3756 cal. per gm., we have as the highest possible value only about 80 cal. per gm. of glycogen hydrolysed. Using the lower value for the heat of combustion of glycogen [48], 3789, the heat available is considerably smaller. In order to produce by such a reaction an amount of energy equal to the heat of oxidation in air, it would be necessary under anaerobic conditions to decompose at least 40 to 50 times as much glycogen as the normal aerobic requirement. Lesser realising this difficulty attempted to meet it by suggesting a further change of a portion of the dextrose, but as we have seen, he was unable to indicate in what way this change occurred.

In the glycogen-lactic acid reaction more definite information is available, Meyerhof [43] having determined the heat evolved by muscle for each gm. of lactic acid formed, and found it to be 390 cal. The maximum difference between the heat of combustion of glycogen in dilute solution (3836 [56]) and lactic acid under the same conditions (3601 [46]) is only 235 cal. The deficit has been explained in various

ways as representing the heat of neutralisation of the lactic acid by protein, and various other secondary changes. The most recent discussion of this question is due to Meyerhof and Suranyi (49), and may be taken as indicative of the present involved state of the problem. For the immediate purpose it is sufficient to know that the high value for the heat output when lactic acid is formed is capable of theoretical explanation.

Hill and Hartree (23, 24) have compared the heat given out by a series of isometric contractions—in which all the liberated energy appears as heat—under anaerobic conditions, with the heat evolved when air is readmitted after such a series of contractions. The first value corresponds to that measured by Meyerhof (43) and consists essentially of the heat of formation of lactic acid from glycogen, and the heat of neutralisation of the lactic acid; the second is the heat given out by the burning of one-fifth of the lactic acid—or its carbohydrate equivalent—less the heats required to free the lactic acid from its salts and to reform four-fifths of the glycogen involved in the original breakdown. These quantities proved to be almost equal, so that under anaerobic conditions the formation of lactic acid must liberate at least half the energy available in the normal oxidative cycle.

A similar result can be reached by calculation from the heat of combustion of glycogen and the value obtained by Meyerhof for the heat production accompanying the formation of 1 gm. of lactic acid under anaerobic conditions, viz. 390 cal. Thus if we consider first the normal cycle in the presence of oxygen, assuming the rough relationship of 1 gm. of carbohydrate burnt for each 5 gm. broken down in the first instance to lactic acid, we have for every 5 gm. of glycogen originally involved a total output of heat both anaerobic and aerobic equal to the heat of combustion of the 1 gm. of glycogen disappearing, i.e. 3836 cal. Under anaerobic conditions, without the oxidative recovery, the first part of the cycle will produce from the 5 gm. of glycogen, 5 gm. of lactic acid, with a corresponding heat production of 390 × 5 cal., i.e. 1950 cal. Thus the anaerobic heat calculated in this way is approximately half the total heat. Moreover the oxidative heat appears to be merely a waste product of recovery, except in so much as it maintains the body temperature. The anaerobic metabolism viewed in this way appears as efficient as the aerobic, with the marked difference that five times as much glycogen is broken down, and without recovery the energy in the lactic acid so produced must be wasted.

Thus the lactic acid cycle is peculiarly efficient as a source of anaerobic energy, since not only does it supply the same immediate energy for the vital processes, whether oxygen is present or not, but on the readmission of air the tissue returns to a normal condition with the liberation of the oxidative heat in full. Hence by this means an animal can undergo a limited period of anaerobiosis, without an interference with its normal energy supply, or without the expenditure of any excess energy in the process.

VI. THE EFFECT OF OXYGEN LACK ON NERVE TISSUE.

The profound effect of oxygen lack upon the nervous system of the higher animals has not so far been discussed owing to the special circumstances involved. Any marked change in the nerves of an animal under examination will be immediately apparent in its response to external stimuli, and in consequence the actually recorded observations will be largely the result of variations in the nervous tissue.

The animals so far examined fall into two groups; the first consists of the earthworm and the various intestinal round worms, in which movement continues and a normal response to stimulus is obtained in the complete absence of oxygen; the second is made up of all the more highly differentiated animals, whose nerves lose their excitability and power of control at low oxygen tensions.

This latter group automatically divides itself again by virtue of the part played by the nerves in supplying oxygen to the body. An organism which depends upon a delicate circulatory system for its oxygen supply, cannot recover after oxygen lack, because the nerves have no longer the power to set in motion the means of supply. On the other hand, if only automatic physical effects are involved in the oxygen supply, when air is readmitted, it is immediately carried to the various parts of the organism and recovery commences. The nerves get the same supply as the rest of the body and as they recover excitability returns. In the cockroach this phenomenon is very striking, the insects remain completely motionless long after oxygen is available, only regaining the power of movement as the oxygen debt is discharged.

So far as the author is aware no explanation has been given of the varying susceptibility of the nervous system in different animals to oxygen lack. An examination of the nervous anatomy of different groups of animals in the light of their behaviour under anaerobic conditions, might possibly indicate those structures which are responsible for the collapse of the higher animals. It appears that at some stage in the evolution of the nervous system a type of cell arose, which unlike all the other living cells is immediately dependent on molecular oxygen for its function. On the other hand it does not die when oxygen is not available, and slowly recovers its excitability as lactic acid is removed from the surrounding tissue. The presence of such cells must be a grave disadvantage to the animal, and it is difficult to imagine why all trace of resistance to oxygen lack has been lost, and what the corresponding advantages can be.

Gerrard has recently published a series of investigations into the oxygen requirements and anaerobic behaviour of isolated nerves. He had previously shown whilst working with A. V. Hill [10, 14, 15], that when a nerve is stimulated, heat is given out in a short and intense phase associated with the carriage of the impulse, and in a weak delayed output lasting for a considerable time. From these results Gerrard expected the mechanism in nerve to be similar to that in muscle, and hence to depend on the lactic acid cycle. In order to confirm this view he examined the heat production in nerve under anaerobic conditions [11], and found that the total heat given out slowly decreases from the time when air is excluded. The recovery

heat however was still present and only disappeared at the same rate as the initial heat. Nerve must therefore be unlike muscle in having a recovery heat that is not oxidative.

Recovery after admitting oxygen is in the case of nerve merely a renewal of full activity, the initial and delayed heats increasing together.

Unable to reconcile these facts with the theory of lactic acid production, Gerrard in conjunction with Meyerhof (16) measured the lactic acid in nerve under various conditions. They found no lactic acid formation in nerves at rest in oxygen, but in nitrogen lactic acid was produced, and continued to be so, until the supply of carbohydrate failed. Stimulation in nitrogen causes a slight increase in lactic acid production, but not at all commensurate with the increased oxygen intake on stimulation in air.

The accumulation of lactic acid does not seem to check further glycolysis, and the acid is not removed, or only very slowly, when air is again admitted. Gerrard and Meyerhof suggest therefore a lack of glycogen resynthesis and probably of lactic acid oxidation in the case of nerve.

Gerrard carried the work still farther (12, 13) by means of manometric measurements. Meyerhof (47) has shown that added lactic acid causes a rise in oxygen intake in muscle due to resynthesis of glycogen, but Gerrard could find no such change in nerve, thus adding to the evidence in favour of the absence of resynthesis during recovery.

A nerve which had been in nitrogen was found to have an oxygen debt equal to about two-thirds of the resting metabolism during the recovery period. As this did not appear to be involved in the oxidative removal of lactic acid, Gerrard argues that nerve must carry on in an inert gas by virtue of a store of oxidising substance, which is replaced during recovery. If this were the case carbon dioxide should be formed during the anaerobic period, not merely liberated from bicarbonates. By determining the total carbon dioxide present before and after a period of anaerobiosis, Gerrard succeeded in showing that about one-tenth as much carbon dioxide had been formed as during an equivalent aerobic period.

It would appear therefore that a good case had been made out for the use of stored oxygen by nerve during anaerobiosis, a view originally advanced by Fröhlich (8) in 1904 and later supported by Gottschalk (17), but it is difficult to reconcile this view with the production of lactic acid under conditions of oxygen lack. Several reactions may be going on side by side in the nerve, each contributing a little to the sum total of the energy, and it must be left to further experiment to decide what part these reactions play.

From the point of view of the whole animal it is far more important to remember that the nerve fibre only loses its power of conduction slowly. That part of the nervous system which is influenced immediately by oxygen lack must have for its function a different type of mechanism, and obviously cannot go into oxygen debt. As to the nature of this mechanism it is impossible at the present time even to speculate.

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THE CONTRACTILE VACUOLE

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(With Seven Text-figures.)

CONTENTS.

IN the following pages I have made no attempt at exhaustively detailed presentation of the problem of the contractile vacuole, involving as it does a multiplicity of forms most of which are only slightly known and have been but little studied. I have preferred rather to discuss a few better known and more exhaustively studied kinds, since in this way the general problems are no less advantageously indicated. For such details of other known forms not mentioned here, the student is referred to Dujardin, Butschli, Pritchard, Kent, Minchin, Lühe, Burian and Calkins, together with some recent papers which contain fuller historic reference. It has seemed better to make the effort to give fuller accounts of a few forms in a comparative way than to attempt a well-nigh impossible task.

I. TERMINOLOGY.

Any effort at uniformity of terminology is commendable, if the terminology works. My desire to achieve it, however, has not led to much success. The name "contractile vacuole" has more or less annoyed various students of the protozoa, many of the earlier observers (*e.g.* Carter) preferring "vesicle" to "vacuole," on the ground that a vacuole, to deserve the name, should have nothing in it. But then, neither should "cell," or at least nothing perhaps but bad air. Vesicle sounds better if one has been using vacuole repeatedly, and sometimes I use it. It then means the same thing. Whether to say "pulsating" or "contractile" should depend, one would think, on whether one is describing repeated behaviour of a persistent structure, or the serial appearance of similar organs doing the same thing, contracting. Haeckell suggested the shorter term of "systolette," and Pütter bravely followed him, but apparently did not succeed very well in making it stick, as nobody else, as far as I can find, has emulated his example. Perhaps Haeckell would have

had better luck with "diastolette." The difficulties in which one lands when one tries to be rigidly logical, are amusing, but harmless. Pütter, for example, adopted "systolette" for the sake of brevity (he was talking about *Paramecium*) but when he had to talk about the rays, or radial canals, or canals, he found himself calling them "Bildungsvacuole und Zufuehrungskanäle." Of course he was at a disadvantage linguistically. Taylor succeeded much better by using symbols V_1, V_2 and V_3, but this works only for *Euplotes* at present. I have therefore ignobly followed the course of least resistance, and shall talk about contractile vacuoles, and the radiating canals ("l'étoile" of the French protozoologists) of *Paramecium* as the canals. Since the question of pulsation is still open for many if not most forms, it will be answered for those discussed without any short cut.

The partition of the contractile vacuoles of *Euglena* which takes place, according to Klebs (1883 through de Vries 1889) during fission, raises this structure to the dignity of a permanent organ in the sense of de Vries (1889), and the constancy of this "organelle" reflects the possibility of others being in the same case.

For the purpose of the present paper it must be observed that other vacuoles, those containing solids, may discharge their contents to the exterior and behave primarily as contractile vacuoles *in sensu proprio*. The food vacuoles of *Paramecium* thus expel their contents, but at a fixed locus. I have observed the same, a matter of common experience, for *Amoeba*, but without a fixed locus, and the discharge of oil droplets by myxamoeboid organisms is a similar procedure (Gilbert, 1928).

II. SYSTEMATIC OCCURRENCE.

The contractile vacuole is recognised as occurring alike in plants and animals of less differentiated condition. It characterises the zoosporic condition of algae and fungi (one or two vacuoles) as also those which retain this form through life (Volvocineae)[1]; the zoospores and plasmodia of the Myxomycetes (Mycetozoa) and the protozoa in general. Contractile vacuoles also occur in the gametes of *Spirogyra* during conjugation leading to their condensation to form the zygote (Lloyd, 1928) and it is likely that they may be found in other Conjugales in like situation. That they also play a rôle in Metazoa and Metaphyta is not yet admitted, but there is some reason to believe that they do (Hartog, 1889, Ludford, 1925). Pfeffer[2], it is true, has not cared to sharply separate the vacuoles in contracting tissues of the pulvinus from the contractile vacuoles of the algae, etc., but does so on the general ground that they are not constant in volume. It is not excluded, however, that the escape of liquid from the cells of these or other organs may be by means of contractile vacuoles rather than by changes in permeability of the cell membranes. As Pfeffer points out, their systolic and diastolic movements may be so slow as to escape attention; or they may be small or otherwise difficult of observation.

In all known cases, the contractile vacuole is positively correlated with either the absence or non-rigidity of the cell wall or with the internal displacement of

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[1] If the compound zoospores of *Vaucheria* have contractile vacuoles they have been overlooked (Klebs, Strasburger), and the same may be said for the *Chytridiales* (Karling, 1928, *Amer. Journ. Bot.* **15**, 32–42). Hazen (1899) expressly denies its occurrence in *Sphaerella lacustris*.
[2] See his *Physiology of Plants*.

the protoplast therefrom. A typical case is afforded by *Amoeba*. The occurrence of contractile vacuoles in shelled forms such as *Arcella* has no significance in this connection since the shell is a housing, but not a complete investment. *Vampyrella* has contractile vacuoles even when encysted, at least for some time after passing into this condition (Lloyd, 1926). The various algal zoospores are stated by West (1916) to be naked protoplasts and were observed to show amoeboid capacities (Klebs). The absence of contractile vacuoles from the megazooids of *Sphaerella* (Hazen) supports this generalisation, since a cellulose investment occurs. That they were not observed in the micro-ozooids may be an oversight, or there may be here also a cellulose membrane, though their change in shape, described by Hazen, may indicate its absence.

Similar relations are exhibited by *Brachiomonas* and *Lobomonas* (Hazen, 1922 *a*). Their absence from the former, an organism invested with a cellulose wall, is regarded by Hazen as a mark of high specialisation. We might however say that the mark in question is the cellulose investment, the absence of contractile vacuoles being consequent. That this absence is surely correlated with a marine environment is doubtful since contractile vacuoles certainly occur in some marine forms as *e.g. Chlamydomonas*; but *Lobomonas* (Hazen, 1922 *a*, p. 87) with two alternating contractile vacuoles, seems also to have a cellulose wall, though its extreme delicacy in young individuals may approximate mechanically to near-absence. The membrane becomes thicker with age (Hazen) and the contractile vacuole may then also disappear. Hazen himself expresses the belief that "The form development of *Lobomonas*, *Brachiomonas*, and *Pteromonas* must be essentially amoeboid for a brief period during the organisation of the daughter cells, and we are justified in assuming that their lobes and excrescences are the expression of the same non-homogeneous organisation of the protoplast as is characteristic of *Amoeba*." This is a view which goes some distance in supporting the theory (Hartog, 1889) that the contractile vacuole is connected with absence or minor rigidity of the cell wall. When a slight or irregular wall is present, the maintenance of equilibrium (as expressed by shape) may be quite dependent on the expulsion of water. The persistence of contractile vacuoles as the cell wall becomes more rigid with age may account for the shrinkage "due" as Hazen suggests "to loss of a certain amount of water from the protoplast." The living protoplast of *Lobomonas* as it ages retires from immediate contact with the lobulated cell wall. If this is true, the behaviour would be quite analogous to that of the gametes of *Spirogyra* (Lloyd). In *Volvox globator* there are two to six contractile vacuoles whose contractions show no rhythm. In *Volvox aureus* there are two only and the contractions here also "ne sont pas concordants" (Janet, 1912).

Among animals, contractile vacuoles are highly characteristic of the rhizopods, flagellates, and ciliates, but, though they occur in some marine forms, they seem to be more frequently absent. Their number may vary and this even in the same organism (*Amoeba*, *Arcella*, etc.) or is usually constant, *e.g.* two (or more, Hance, 1917) in *Paramecium*, one in *Euglena*.

III. STRUCTURE.

In order to simplify discussion, the more extensively studied forms will be discussed concretely, omitting the introduction of many details which would add little or nothing to a general résumé.

Amoeba. The contractile vacuole in *Amoeba* is a "roving" vacuole, that is, its position[1] is not fixed so long as it does not become attached to the ectoplasm, which happens just previous to the expulsion of the contents. While free they may be carried forwards and backwards as passive masses comparable to granules, etc. To the botanist who is familiar with the plant cell, there is little question at the moment that the membrane is nothing more or less than such a membrane as lines vacuoles in plant cells. From a biophysical point of view, it is a concentration membrane and has no permanence as an "organelle." "The larger vacuoles...to which the names cavulae and contractile vacuoles are given, are interpreted according to the alveolar theory as due to the flowing together and fusion of·adjacent alveoli (Calkins). This is certainly the case in the formation of a contractile vacuole of *Amoeba* proteus where the beginnings of a vacuole may be watched...and the coalescence of minute vacuoles noted. In a similar way the relatively huge cavulae or pseudoalveoli, characteristic of *Actinosphaerium lichhornii* and of *Radiolaria* may be accounted for" (Calkins, 1926, p. 43).

A typical modern pronouncement of this view is made by van Herwerden (1927) who observed, on the disappearance of the contractile vacuole, that there appears in its place a coarsening of the endoplasmic granules. These granules are to be regarded as ultramicroscopically fine alveoli ("Blasen") for soon in this same place a fine alveolar structure becomes visible while the rest of the endoplasm retains its usual granular appearance. After a few minutes, on the disappearance of this, a few alveolar spaces are seen and thus a number of small vacuoles arise which finally run together to make a single larger one (Fig. 1).
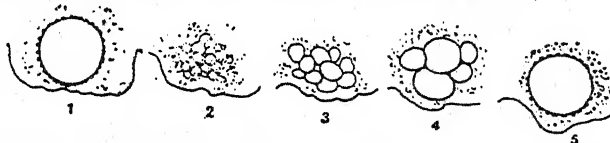

Fig. 1. The vacuole of *Amoeba proteus*. (1) Vacuole before emptying; (2) local net structure in the endoplasm; (3) the formation of small vacuoles; (4) running together of vacuoles; (5) the new vacuole arising therefrom. Drawn from life by van Herwerden (1927).

"There is no doubt that, in the *Amoeba* studied by me, we are concerned with a local change of phases of the granular endoplasm, whereby in a narrowly restricted zone an alveolar structure is temporarily assumed" (Van Herwerden (1927), p. 274).

Van Herwerden finds support in the observations of C. V. Taylor on *Euplotes*. This species Taylor (1923) regards as standing, in point of structure "in the series formulated by Khainsky of the transitional types of vacuoles" about half-way between *Amoeba* and *Vorticella*, the position of the contractile vacuole being constant and its pulsations regular, but lacking canals.

[1] The temporary position in relation to the nucleus (Rhumbler, 1898, p. 227) in *Amoeba proteus* seems not to be of special importance.

Now the evidence in regard to *Paramecium* supplied by Nassonow is rather convincing that the contractile vacuole mechanism is different from that in *Amoeba*, and I shall shortly offer my own observations on this case. I mention this at the moment to observe that in view of differences in structure, any effort to generalise as regards the origin of the contractile vacuole is fraught with danger. For instance, it is not justifiable to use the observations on *Paramecium* made by R. A. Young (1924), correct though they may be in themselves, to argue that Taylor's idea of the vacuolar mechanism in *Euplotes* is unsupported by evidence or necessity. These two organisms may be quite different, as also may be *Euplotes* and *Amoeba*. As we shall see, Taylor's view is in harmony with the observations of van Herwerden, but it seems to me somewhat doubtful if the case of *Euplotes* is as clear in point of evidence as that of *Amoeba*.

Ruth B. Howland (1924) favours the view that the precursors of the contractile vacuole are to be found in certain granules which dissolve to form minute droplets. To this author these granules appeared frequently "to increase in size as though with the inhibition of water, their heavy outline paling out as the centre of the granule becomes clear." It is readily appreciated that these views may state facts as different which are really identical, since "granules" may really be alveoli; or again the alveoli may in some animals or under some circumstances contain fluid and, in others, matter sufficiently non-fluid to be called granules. As will appear later, there is little objective support for an argument which takes granules as a serious consideration, as there is no evidence that granules of this sort play any part in the formation of contractile vacuoles in *Spirogyra*, or in *Paramecium*.

Metcalf (1926) however records "the constant association of the contractile vacuole with a certain group of granules, the new vacuole appearing among the old granules." These he compares with "similar" (?) ones "surrounding the posterior vacuole" of the "excretory" system of tubules in *Protoopalina*. If it is a fact, as Metcalf maintains, that some of these "granules drop into the lumen of the posterior vacuole" and are expelled, it is evident that such at least are not concerned in the origin of the vacuole. Without committing himself finally, the granules in question appear to Metcalf as indicating a possibly renal function rather than that they are concerned in the origin of the vacuole. He nevertheless insists that the repeated reappearance of the contractile vacuole is always "among the same granules which surrounded it before its last contraction." He goes no further than to assert that "there is a constant relation between the excretory vacuole and the group of granules which surround it."

Mast (1926) seems to agree measurably with Metcalf, but his conviction is somewhat weakened by the mobile character of the vacuole which "is usually found floating about in the plasmasol." Miss Howland (1924) however, having studied *Amoeba, in vivo*, has been unable to observe "any massing of granules as described by Metcalf...for granules of various sizes are constantly being carried across the wall of the vacuole and to all parts of the cell in its active cycloses. The random origin of additional vacuoles seems to Miss Howland to introduce further difficulty.

Day (1927) believes that he has observed minute granules in active Brownian

movement "in the immediate vicinity of a contractile vacuole," upon which he speculated as to the "possibility of these oscillatory granules themselves having something to do with the process of vacuolisation," arguing that, at all events, "the area is not gelated." When *Amoeba* is placed in conductivity water its cytoplasm becomes a veritable foam, Day observes; thus it behaves much as plant cytoplasm has long been known to behave on being exposed to even ordinary water, as when the protoplasm of *Hydrocharis* (Pfeffer) or *Vaucheria* is expressed[1]. Whereupon, being "convinced that association of granules and contractile vacuoles is by no means a constant one" and that it "has no specific relation, morphological or physiological, to any localised area," Day accepts Taylor's view above cited as the most plausible, and argues that the original vacuoles owe their origin to "ultra-microscopic droplets of soluble katabolic waste." Day's observation recalls the dictum that every granule lies in a vacuole. Where there is a granule there must be a cytoplasmic surface. Suitable granules can act as centres of condensation for salts, etc., and thus a centre, in the form of a minute vacuole with a crystalloid concentration higher than that of the surrounding cytoplasm may be supplied. It is also possible that the chemical combination of substances can act (Prat and Malkovsky, 1927, p. 346) locally, as suggested by Iljin's work (Anatonosis), in which he found that some salts can quickly increase the osmotic value of a cell by the production of osmotically active substances.

No discussion of the origin of vacuoles can disregard the work of our French colleagues, especially Guilliermond and P. A. and P. Dangeard. To be sure they do not consider the specific problem of the contractile vacuole, but we are at a loss at the moment to know how to dissociate the two categories, and assume that the problem at bottom is the same, as indeed Went (1890, p. 362) suspected it to be from his point of view, arguing support for his thesis of the genetical continuity from the behaviour of the contractile vacuole in *Euglena* (Klebs) during cell-division. It seems, however, antagonistic to this view that the contractile vacuole is formed *de novo* in fragments of *Stentor* (Gruber, A., through Nussbaum, Karsten and Weber) and of *Amoeba* (Hofer, 1889, through Pfeffer, 1890, p. 193).

A certain degree of consonance characterises the views of these students (Guilliermond and the Dangeards) of the problem, so much so that Bowen remarks that his own results "bear out the recent researches" of the authors in question and "go far toward establishing the vacuome as an independent and permanent part of the plant cell system" (Bowen, 1927, p. 188).

But their views are by no means identical. The Dangeards hold firmly to the vacuome (a collective term embracing the whole vacuolar system of a cell) as a permanent, limitable organ of the cell, which does not originate *de novo*, homologising it with the chondriome of animal cells; while Guilliermond takes the opposite view, saying that "the vacuome of vegetable cells is constituted by the colloidal products of metabolism secreted by the cell in the form of substances which being non-miscible with the cytoplasmic colloids, are by their capacity to take up water,

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[1] But de Vries (1889) regards the vacuoles so arising as pathological, such being also characteristic of senescence or death.

transformed by hydratation into liquid vacuoles." Guilliermond identifies the vacuome with the Golgi apparatus and the canaliculi of Holmgren in animal cells, and, without denying that it may pass from cell to cell, insists that it may also arise *de novo*.

These opposed views trace back to de Vries, van Tieghem and Went on the one hand, and Pfeffer (1877, see 1890) and Bütschli (1894) on the other, the former holding to the genetical continuity view, Pfeffer to a purely physiological or indeed biophysical idea, and Bütschli connecting it especially with his alveolar theory of protoplasmic structure. In de Vries' view, the appearance of any vacuole is to be referred to a persistent but inactive tonoplast, in which the already present imbibition water of the protoplasm collects in the inner region. Such water must have an appreciable osmotic pressure, otherwise the droplets so formed would not grow into large vacuoles, says de Vries, who added "this excretion of water at interior points is itself a yet unfounded hypothesis" (p. 351). This criticism points to a factor of the problem common to both schools, namely, the mechanism of water (or other) collection within the vacuole, whether this is formed *de novo* or already present.

It is apparent that so far as observation goes, students differ as to the facts which may be observed. The idea which seems to be gaining acceptance and which aligns with modern biophysical knowledge is that the origin of the contractile vacuole commences as a localised reversal of phases of the endoplasm. That the appearance of even numerous vacuoles really involves phase reversal is doubtful (Seifriz, 1923). The further question is also left unanswered, namely, what leads to the alteration of phases or to the appearance of vacuoles if they have no continual existence?

This question brings us back to the views above set forth, that, whether the vacuole is or is not of *de novo* origin, there must be some sort of a body capable of attracting water. A group of such bodies would supply the conditions for "phase reversal." For ordinary vacuoles it has been shown that such substances stainable with Neutral Red (*inter alia*) occur, and these, however different, satisfy the demand (Scarth, 1926). According to P. A. Dangeard these substances are metachromatic in the first instance; to Guilliermond it differs from one plant to another. Dangeard (1927), however, holds that various substances may occur in the vacuoles. Whether it can be shown to occur, in fact what can at the moment be only postulated, is for the future; but it may be pointed out that apparently no one has succeeded in getting Neutral Red to be taken up *by the contractile vacuole* (Degen, 1905), as I have myself repeatedly verified for *Spirogyra*, *Paramecium* and *Amoeba*. It would seem, at all events, that if in the last named the origin of the contractile vacuole is as has been described, a positive result should be obtainable.

We come to the further question of structure. As to the existence in contractile vacuoles of *Amoeba* of a membrane of any degree of permanence, there is no evidence (Bütschli (1887–9)). The complete disappearance of the membrane at the close of systole and the appearance of a new membrane is scarcely questioned. The fact that it is possible, as Miss Howland found (1924), to indent the membrane with the point of a needle, or that the contractile vacuoles can be isolated in water and

maintain their identity for a long time, is no evidence for a structural membrane; and the wrinkled character of the membrane when subjected to Neutral Red may be taken to mean that this dye has altered the membrane by adsorption, as happens in plant cells when the toxicity of the dye begins to express itself. The contractile vacuoles may arise anywhere within the animal (Day, 1927) and their roving character—an observation too common to need the citation of authors—added to direct observation, seems to leave no doubt of the temporary character of the membrane; indeed, the history of the vacuole from origin to ultimate discharge "makes it difficult to conceive the (contractile) vacuole as so intricate an apparatus as Stempell (1914) describes it." I quote from Seifriz (1921), who, however, goes too far, I think, in putting *Paramecium* in the same category with *Amoeba* in this regard.

That the contractile vacuole can occur in any position is generally admitted for *Amoeba* and many, if not all, amoeboid forms. If this is true, it would be soundly argued that there is accordingly no constant pore or exit mechanism, and we are forced to say that the escape of vacuolar fluid takes place by the breaking of the outer surface and some depth of ectoplasm. The conditions seem to be alike for *Amoeba* and *Spirogyra*. There is, however, some difference, for whereas the bursting of a contractile vacuole in the latter is accompanied by an inward displacement of the cytoplasmic membrane (that is, the whole layer of cytoplasm from wall to central vacuole) I have never seen such displacement in *Amoeba*[1]. In this the discharge of the vacuole is characterised by a rush of cytoplasm (plasmasol of Mast) to occupy the place of the vacuole, unaccompanied by any distortion of the surface except, of course, at the immediate point of exit. There is no relaxation of the ectoplasm about the opening. This may be taken to mean that the ectoplasm is of relatively highly rigid character—Mast calls it plasma-gel—and by way of confirmatory evidence I point out that in the case of a bursting of a food vacuole in *Amoeba* (of the *limax* type) I observed a different procedure, but indicating the same view in regard to the high viscosity of the ectoplasm. From this food vacuole which lay in the tail, the contents were spilled by bursting, followed by a very gradual withdrawal of the ectoplasm, which became cup-shaped, the edges of the cup being gradually withdrawn. The slowness of the process, which occupied several minutes, was significant. In the case of *A. verrucosa*, however, the matter was quite different. The animal behaved at first as if about to divide, the large food vacuole being brought to the thinner isthmus of the body. After some time there was a very sudden expulsion, the contents in a single mass being shot out into the surrounding medium. In this case the food vacuole acted, apparently, exactly like a contractile vacuole. Immediately after the expulsion the two parts of the animal drew together and it moved off.

Thus the very stiffness of the ectoplasm brings about an appearance suggesting the presence of a permanent pore, though the testimony is generally against this for *Amoeba*. E. Frances Botsford (1926) has, however, observed a peculiar behaviour in a sort of eruption of the ectoplasm following discharge lasting four or five seconds.

[1] Rhumbler (1898, p. 263) observed contractile vacuoles in *Amoeba geminata* which explode into the surrounding plasma. This matter needs further study.

If it occurs in similar species it has escaped my observation, but I have seen something similar (and have recorded it in motion pictures) in *Actinosphaerium*. On the collapse of the vacuole it acts, not as a soap bubble but rather as a bubble of some very viscous material would if the fluid escaped through a small opening, that is, the membrane collapses against the floor of the vacuole and appears to rebound. I regard this to be a resurgence of the membrane in folds before it has time to assume minimal area of surface[1]. It seems doubtful that here or in *Amoeba* it is due to a positive tropism toward the spilled contents of the vacuole (Botsford). I have recently observed in *Amoeba verrucosa* that, following systole, which is slow (occupying about 10 seconds) the pore position is marked for several seconds by a papilla which, because of its optical properties, can be very easily seen. It gradually fades away.

Paramecium. Nassonow (1924) has made a very painstaking and exhaustive study of *Paramecium* and some other protozoa by methods of fixation (osmication) and by study *in vivo*. His main purpose was to establish a homology between the Golgi apparatus in the metazoan cell and the contractile vacuole of protozoa, a theoretical question with which we are not at the moment concerned. Apart from this, however, he has offered good evidence that the contractile vacuole in *Paramecium* is a permanent "organelle[2]," and consists of a branched lacuna in the endoplasm lined by a special sort of membrane, one in some way different from that of *Amoeba*. I have myself studied this organism alive at considerable length before having seen Nassonow's paper and find myself in complete agreement as to the permanent character of the membrane. He says: "The excretory apparatus of *Paramecium* consists of a reservoir ('the pulsating vacuole' of Authors) and of canals immediately connected therewith, 1–11 in number, averaging 6. Both reservoir and canals are provided with a membrane (Müller, 1856) which, thinner in the reservoir, passes over directly and continuously into the thicker membrane of the canals." Each canal has three regions, the connecting piece, the ampulla and the distal end-portion. The last extends far into the endoplasm, much further than can be traced *in vivo* (Nassonow). Nassonow was able to osmicate the membranes and thus to bring them into view; otherwise they escape observation. If this account, which is further supported by the observation of R. A. Young (1924), who also worked with fixed and stained material, is correct, the question of origin, as required for such forms as *Amoeba*, is not pertinent. They remain, and, after the animal has been treated with alizarin blue, can be dissected out (Howland, 1924), and though this fact alone does not prove the permanent character of the membrane (the vacuole of *Amoeba* can also be set free, as can plant cell vacuoles), the freed vacuoles with their radiating canals have in their appearance a confirmatory value of a sort. As Miss Howland says, the membranes are probably "set" by the stain, which occurs, according to my own observation, to the vacuolar membrane in the parenchyma of *Opuntia* when the cells become moribund after long exposure to the stain

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[1] Stokes (1893) described the behaviour quite clearly, but believed that the folds were villi, each a tube with a pore, through which the fluid escapes, and that the membrane does not burst.
[2] Organula: Möbius, through Bütschli, 1887–9.

(Neutral Red) and of *Spirogyra* gametes (Lloyd, 1928). Weatherby (1927) found, on injecting Nessler's reagent into the cytoplasm in the vicinity of the contractile vacuole (*Paramecium*), that the cytoplasm surrounding the contractile vacuole "was dissolved but the membrane around the vacuole remained intact for a short time." Lloyd and Beattie (in press) have been able to show the attachment of the central vacuole to the pellicle by the use of Neutral Red, which, in proper concentration, causes the endoplasm to shrink from the pellicle, leaving the neck of the contractile vacuole attached to and dimpling the pellicle (Fig. 4). Physically, however, it behaves like contractile vacuoles in general (as Pfeffer long ago described). Under the action of eosin (1 : 200) the vacuoles gradually enlarge and finally fuse into one, the canaliculi retaining their individuality and undergoing diastole and systole the while. The fusion is rapid and complete and looks in no way different from fusing vacuoles in *Spirogyra*.

Correlated with the permanent character of the contractile vacuolar apparatus, and with its constancy of position, the vacuole opens at one point in the ectoplasm. This appears *in vivo* to the eye as a shining granule or "circlet" (probably *ca.* in the centre of the "pale spot" of earlier observers—C. V. Taylor, 1923) which I have myself observed. Khainsky (1910) thought the pore temporary but recognises the permanence of the region of the ectoplasm concerned in evacuating the vacuole by calling it the "papilla pulsatoria[1]." There have been several observers, including Rhumbler (1898) and Lancaster (1909), who have taken the view that the pore is "definite if not persistent" (Taylor), that is, I suppose, an opening capable of closure as by a mechanism of some kind. In Zenker's (1866, through Bütschli, 1887–9) view, this was a secreted mucilage which sealed the opening during diastole.

Euplotes. In his account of *Euplotes*, Taylor alleges that, on systole (which is complete) of the contractile vacuole ("V_1") a group of secondary vacuoles (Group "V_2"), adjacent to the contractile vacuole before systole, now moves into the position just vacated, where they coalesce to form another contractile vacuole. Meanwhile a third group (Group "V_3") of vacuoles forming a zone beyond the limits of the secondary group has formed, which, the individual vacuoles growing the while, moves forward into the position of the secondary group. The contact of the contractile vacuole with the ectoplasm occurs at a constant point, the "papilla pulsatoria" of Khainsky. The change of the coalesced group V_2 to a perfectly smooth contoured vacuole (having minimum area of surface) is slow, and vestiges of contiguous surfaces may remain as internal ridges, etc., inside the surface of V_1.

By experimental manipulation Taylor was able to induce the formation of supernumerary vacuoles in the general region of the food vacuole, but never in the "area left (hand) of the cytostome." Two possible origins for these are suggested, viz. (*a*) the fusion of smaller pre-existent vacuoles, or (*b*) *de novo*. As bearing on the latter mode of origin, Taylor was able sometimes to initiate vacuoles by the microinjection of solutions of methylene blue, and salts of Na and K. These

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[1] I cannot accept Khainsky's description of a depressed area above the vacuole, a condition evidently due to the fixative.

vacuoles may unite with others (V_2 or V_3) to contribute to the functions of V_1 and thus their contents could be expelled. Sometimes they diminish in size—probably because of the low concentrations used (1 : 10,000 to 1 : 50,000).

The origin of the contractile vacuole (V_1 of *Euplotes*) in *Euplotes* is, however, to be seen in the confluence of the members of Group V_2, and is compared by Taylor to similar phenomena observed by Chambers (1917) in marine ova, in which viscosity changes (indicating sol-gel reversions) accompany the appearance or disappearance of astral rays. Similar reversions have been described by Seifriz.

The obvious comparison is with a reversal of phases in an emulsion, in which the volumes of the suspensoids of a watery internal phase are made to grow until the stability of the system is reduced, resulting in their coalescence into a smaller number. It would be supposed that during increase in volume of the internal phase the viscosity of the system would increase if the suspensoids were closely packed. If not the changes in question might eventuate without any changes in viscosity.

In attempts to explain the origin *de novo* of vacuoles the premise is tacitly or overtly assumed that there must be a fluid-attracting centre which will furnish, in the case of water vacuoles, a concentration of (watery) solution higher than that of the surrounding cytoplasm. Khainsky (1910) attempted to trace the origin of granules (Exkretkörner) to the food vacuoles (*Paramecium*) through whose wall he thought them to migrate. The outward movement of granules from a vacuole is, however, not easy of comprehension, and, as Khainsky's studies were made from fixed and sectioned material, one cannot but hesitate to accept his views.

I have however observed something of this kind to take place in the special case of *Vampyrella*. From the large receptive vacuole (formed by pressure from the bursting food cell (*Spirogyra*), the food material, consisting more obviously of rounded masses of swollen chloroplast, and less so, of cytoplasm and nucleus, certainly does find its way into smaller food vacuoles which now lie in the cytoplasm of the animal (Lloyd, 1926). The only satisfactory explanation of what has happened is that the contraction of the whole animal obliterates the receptive vacuole, the wall of which envelops severally the projecting masses of food material and is left in part embracing them; that is, the passage of food masses, granules, etc., into the cytoplasm is possible only by virtue of the disappearance of the vacuole itself. This occurs, quite obviously, when the central vacuole of the *Spirogyra* gamete finally disappears by condensation through the activity of contractile vacuoles. The food vacuoles in *Paramecium*, however, remain and expel solid detritus, left from digestion, through the anal pore. That, previously, some solids have escaped through the membranes seems incredible. If, however, only solutes thus escape, then we are confronted with the question of how such a local concentration can occur to afford the physical basis for vacuole formation, viz. a higher concentration of solution at one point. Those who, with Dangeard, have approached this question from the point of view of general cytology, have answered the question by stating that in the cytoplasm occur granules which attract water. P. A. Dangeard specifically calls these mitachondria which, on taking up water, becomes the chromidium, a colloidal solution contained in the vacuoles (see above).

Some observations of my own on *Elodea* lead me to question the universality of Dangeard's view. One may observe that, in the youngest embryonic cells of the stem apex and elsewhere, there occur granules. These display Brownian movement in amplitude which are inversely as the size of the cell, and it is easily apparent that these granules lie in the older and ageing cells in the vacuoles. But I observed no evidence that they are hydrophylic. They do not swell. Nevertheless it must be apparent that solid particles, no matter how small, and fluid particles insoluble in water, must, by their very presence, furnish surfaces of cytoplasm, within which, by adsorption in the solid phase on the surface of the enclosed particles, high concentrations may arise. Dangeard does not of course assert that the hydrophylic substance is identical everywhere, but he postulates, at least, this property. That, however, solid particles may appear and constitute a centre about which a vacuole may originate is indicated by the appearance and growth of calcium oxalate in embryonic cells. It seems clear that the incipient vacuole is attributable to the precipitation of this insoluble salt, at first as a globoid of supersaturated solution, about which a cytoplasm-enclosing surface must thus arise. It has been believed that particles which display Brownian movement are always enclosed in minute vacuoles—the idea being that these were large enough to allow some, if little, movement. This is not the same as believing that vacuoles always contain solid particles, but we find this much agreement that a vacuole must always contain something besides water (aside from those which obviously contain water-insoluble fluids).

IV. BEHAVIOUR.

The behaviour of the contractile vacuole in Paramecium. This evidently complex mechanism has been studied by a goodly number of observers[1] with surprisingly varied accounts and interpretations, distributed in time during the last 80 years, and it is a curious fact that the rejection of error by later workers has led also to rejection of the truth.

Thus Müller (1856) and Claparède and Lachmann (1858–61) and others denied Schmidt's belief that there is a pore, through which he maintained that *both* influx and outflow take place. Apart from the denial of the presence of a pore (O. Schmidt) the descriptions of Müller and of Claparède and Lachmann were otherwise and on the whole correct as descriptions of the movements of the vesicle and its rays. Those observers held that the "systole of the rays is synchronous with diastole of the vesicle into which the rays " empty all at once or more or less individually or in groups. Shortly before the vesicle effects its beat (systolic) it becomes smaller, whereupon the rays are filled; then systole ensues, the vesicle quite emptying itself, the rays becoming further expanded, in which condition they remain a short while. They then suddenly empty themselves and the vesicle expands. This expansion is not gradual in *Paramecium*, as in many Infusoria, but sudden, due to their active contraction " (Müller, 1856). Lachmann's description (1857, p. 224) differs a little from the above in a quantitative sense, since he says

[1] For the earlier historical period see Pritchard's *History of Infusoria*.

"by the sudden contraction of the space (*i.e.* the contractile vacuole) the rays *instantly swell* (italics mine) into a pyriform commencement close to the position of the contractile vesicle which has disappeared." The rays are reduced again on diastole of the contractile vacuole. The idea which dominated these accounts was that of backward and forward movement of fluid from the contractile vacuole into the rays. "La vésicule contractile, c'est à dire le cœur, se contracte et chasse le liquide circulatoire dans les vaisseaux, qui, par suite, se distendent. Puis les vaisseaux se contractent à leur tour, soit activement, soit par suite d'une réaction des parois du corps, et chassent de nouveau le liquide dans la vésicule. C'est un mouvement de va et vient continuel..." (Claparède and Lachmann, 1858, p. 51). As late as 1893 Schneider was a protagonist of this view.

These foregoing were supported by Lieberkühn with somewhat more explicit wording. "A little *before* we observe *the commencement of systole, the vessels begin to expand slowly* (italics mine) at points distant about one diameter of the contractile vacuole from the surface of the latter, to many times their original size." During the first half of diastole the canals empty again into the contractile vacuole. Lieberkühn, however, denied that there is any return flow into the rays, so that, while Müller, Claparède, Lachmann, and Lieberkühn agreed on the rhythm of events, the latter differed on interpretation. Carter (1861) made the suggestion that the expanding of the rays was due to "ponding back" the fluid during early systole of the contractile vacuole. He seems not to have apprehended Claparède and Lachmann quite correctly as he attributes to them the view that rays become filled immediately afterwards, *i.e. after* systole of the contractile vacuole. This is not quite what they said. Carter, however, by the use of suspended pigments (China Ink) satisfied himself of the outflow and the absence of inflow through the pore of *Brachionus*, as Jennings did much later for *Paramecium* (1904), while Stokes (1893) made precisely the same observation by watching the displacement of "bacteria and similarly minute bodies" by the "puff" of liquid. See also Rhumbler (1898, p. 257).

Since in Carter's opinion (and correctly) the systole of the contractile vacuole is accompanied by escape of fluid, he seems to have argued that the idea of a return flow into the rays is a mistaken one, and this conclusion seems to have dominated till the present time. Maupas (1883), while having negative views about the membrane[1], gave the same sort of account of movement as Lieberkühn, particularising in saying that a little before the systolic movement of the contractile vacuole takes place, the canaliculi commence to fill in the form of elongated pears, after which he goes on to say that inasmuch as the canaliculi are systolic during early diastole of the contractile vacuoles, they are simple afferent conduits. Following Maupas, this relative behaviour of the rays and contractile vacuoles during the systole of the latter has been lost sight of. Stempell (1914), for example, says that "as soon as the contractile vacuole empties itself, the ends of the contributory canals swell up," a statement which betrays lack of critical inquiry into the precise routine of events.

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[1] For a historical summary of detailed views *re* the presence of a membrane, etc., see Taylor (1923).

Stempell is here identifying the canals with the "Bildungsvacuole" particularly with the events described by Khainsky (1910, p. 24): "Durch den allmaelichen Druck der sich ansammelnden Fluessigkeit werden die Plasmalamellen, die sich zwischen den einzelnen Bildungsvacuolen und Zufuehrungskanälen befinden, gesprengt und letztere fliessen bei ihrer Beruehrung in einer gemeinsamen Vacuole zusammen. Die neugebildete Vacuole nimmt wieder an Umfang zu" (Fig. 2).

According to Khainsky, Pütter (1904), Fig. 3, and Stempell (1914), Fig. 2, the flow of fluid is always from the canaliculi to the contractile vacuole. Pütter says (1904, p. 144) Phase 5 (Fig. 3) is marked by the disappearance of the canals ("Bildungsvacuole und Zufuehrungskanäle") by the concurrence of which the vesicle arose,
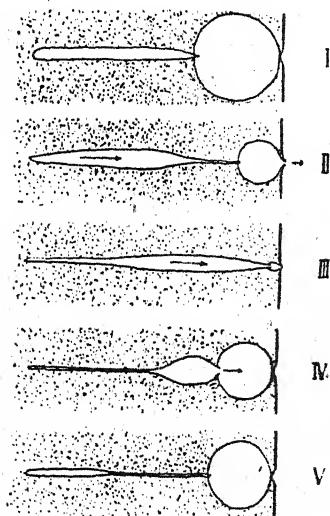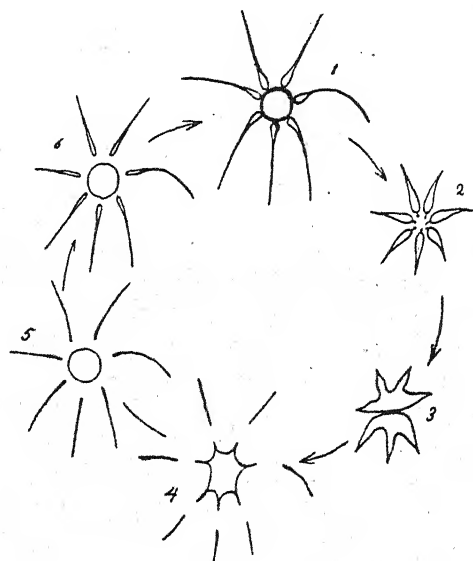

Fig. 2.   Fig. 3.

Fig. 2. Schematic representation of the mechanism of diastole and systole of the pulsating vacuole of *Paramecium*; only one canal is shown. After Stempell (1914).

Fig. 3. The progress of events during the cycle of behaviour of the contractile vacuole and canals in *Paramecium* represented in six phases. (According to Pütter, 1904.)

so that this is now surrounded by a zone in which no canals subsist. These rather form themselves anew distally. Phase 6 shows an advance over Phase 5 only in that the canals have become longer, their proximal ends have come closer to each other and at the same time somewhat swollen. The changes through which Phase 6 goes over into Phase 1 are apparent, and therewith the cycle is completed. It should be noted that from Phase 5 to Phase 1 an enlargement of the vacuole takes place which is conditioned not by the emptying of the fluid out of the canals, but by the direct uptake of fluid out of the surrounding plasma.

It will be seen that the diagram afforded by Pütter, herewith reproduced (Fig. 3), can be otherwise explained by saying that the enlargement of the canals

during Phases 5, 6, 1 is due to the reflux of fluid from the contractile vacuole into them. Nevertheless with both the descriptions and inferences of Stempell and of Pütter, Nassonow wholly agrees (1924, pp. 454 and 459). I quote his own words: "Nach der Entleerung und dem völligen Verschwinden der Vakuole werden in der ihr anliegenden Gegend 5–7 radial gelegene Kanäle deutlich sichtbar (2, Pütter's diagram, Fig. 3). Ihre, dem Zentrum zugewendeten Enden sind stark geschwollen und zwischen den Kanälen ist keine Spur von einer Verbindung zu bemerken. Die geschwollenen Enden nähern sich, fliessen ineinander und bilden eine neue Vakuole, in die nun die Flüssigkeit aus den Kanälen einströmt. Danach verschwinden die Kanäle vollständig (3, Pütter's diagram, Fig. 3), und erst später beginnen sich an ihrer Stelle neue Kanäle zu bilden (4, 5, Pütter's diagram, Fig. 3), welche nach der Entleerung und dem Schwunde der Vakuole an ihren Enden Anschwellungen entstehen lassen und auf diese Weise den Pulsierungszyklus vollenden." Further: "Die Membran der Endabschnitte stellt im Innern des Kanals ein in bezug auf das Plasma hypertones Medium her, was das Eindringen des Wassers durch die Membran hervorruft. Letztere muss hier die Rolle einer semipermeablen Membran spielen. Im Innern entsteht ein gewisser Druck; dieser bewirkt das Aufblähen der Ampullen und das Auseinandergehen der Wände des Schaltstückes, welches die Funktion eines Ventils hat. Die Flüssigkeit strömt längs dem Schaltstück in das Reservoir. Hier ist der Druck nicht genügend stark, um die Flüssigkeit in den Kanal zurückzuwenden, reicht aber aus, die dünne Pellicula der 'Papilla pulsatoria' zu zerreissen und das Reservoir zu entleeren. Somit kann man sich den ganzen Apparat als Pumpe, die mit zwei Ventilen[1] versehen ist, vorstellen."

It appears to me therefore that later authors have overlooked what was apparent to some of the earlier ones, namely, that diastole of the canaliculi is synchronous with the early phase of systole of the contractile vacuole, and does not follow it, and the time relations seen in the rate of diastole both of the contractile vacuoles and canaliculi have been quite generally neglected[2]. From the observations of these time relations it emerges (according to Lloyd and Beattie) that diastole both of the contractile vacuole and canaliculi consists of two periods, an earlier period of rapid expansion of the vacuole due to rapid mass inflow of fluid, and a later period of gradual expansion due to diffusion of liquid. The rapid state of diastole during the earlier period precludes the idea that it can be caused by diffusion. Further, a rapid diastole of the canaliculi always precedes appreciable systole of the contractile vacuoles, or, better, is synchronous with early systole of the contractile vacuoles; and systole of the canaliculi is always synchronous with the early period of diastole of the contractile vacuoles (Fig. 5). These relations cannot be properly understood without admitting that there must be a reflux of fluid from the contractile vacuole into the canals during the very early phase of systole of the contractile vacuoles. There is also an outflow of fluid from the contractile vacuole during the later period

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[1] Pütter's idea of valves being present at the entrances of the canals into the vacuole and at the mouth of the vacuole may be a "nahe liegender Gedanke" (1904, p. 460), but, if Lloyd and Beattie (1928) are right, it is not true of the former, and therefore may not be true of the latter.

[2] Schneider (1893), though wrong in denying the expulsion of liquid, did observe that "the vesicle expands suddenly at first, then slowly."

of systole of the contractile vacuole following the diastole of the canaliculi, as Lloyd and Beattie have proved by the pigment method used by Carter, Jennings, and probably many others. There is accordingly a volume discharged at late systole[1] of the contractile vacuole, and a residual volume that is forced back (this occurring during the earlier phase of systole of contractile vacuole) into the radial canals. I find in the literature only one statement in this sense, which seems
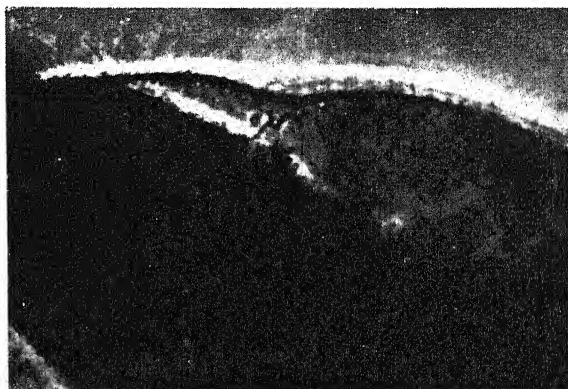

Fig. 4. *Paramecium* treated with Neutral Red 1 to 600. The cytoplasm has withdrawn from the pellicle, the neck of the contractile vesicle being drawn out by the receding cytoplasm, and dimpling the pellicle.
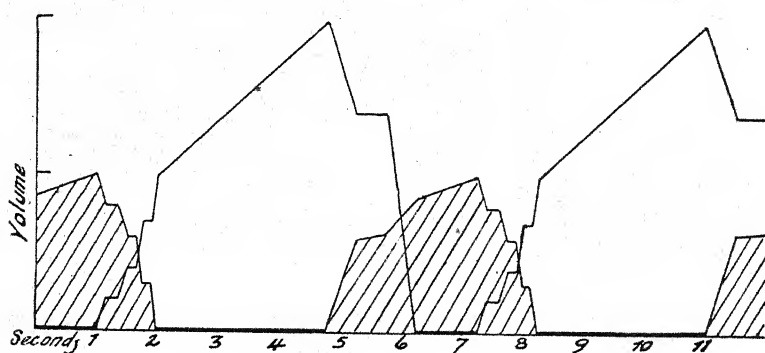

Fig. 5. Diagram showing the time relations of diastole and systole of the contractile vacuole and the canals (hatched) of *Paramecium*. (Lloyd and Beattie.)

to have been quite generally overlooked though cited by Bütschli, namely, that by Fr. Stein (1859), who endeavoured to show that in *Paramecium* at least a part of the fluid flows back into the canals and the rest is expelled (Bütschli, 1887–9, p. 1450). Degen (1905), in *Paramecium caudatum*, observed that "die Canaliculen sich oft mehrere Male entleeren und wieder fuellten, bevor die Systole erfolgt." He does not say anything about how the canaliculi were filled. Maupas (1883), too, saw a

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[1] Samuelson (1857) gave a similar account for *Glaucoma scintillans*.

repeated occurrence of accessory vacuoles during the same diastole in *Paramecium Aurelia*, but a careful reading of his description betrays the fact that he did not regard the canaliculi as deriving their first flush of fluid from the contractile vacuoles. He says: "The canaliculi are therefore simple afferent conduits, moreover I have never seen the liquid of the vacuole re-enter them."

Nassonow's figures[1] can be interpreted as well one way as the other, made as they were from fixed material. Thus, when, at the beginning of systole of the contractile vacuole, the canals become filled, the contractile vacuole is nearly or truly spherical, while at the beginning of diastole of the contractile vacuole it usually is irregular. Thus, Nassonow's Fig. 40 is probably that of early systole of the contractile vacuole and synchronous diastole of the canals; his Fig. 42 represents early diastole of the contractile vacuoles and systole of the canals, precisely the opposite of his own descriptions. It is then of very great interest that we find Wrzesniowsky, in 1869, recording for *Enchelyodon farctus* that "as soon as the contractile vacuole begins to contract there appears on its upper surface numerous droplets which enlarge as the contractile vacuole contracts, at first slowly till about half emptied and finishing suddenly leaving behind numerous minute vacuoles." This passage, quoted also by Degen, leads him to say that he regards the sudden contraction as the true systole, the earlier reduction being caused by the formation of the accessory vacuoles, finding moreover that this view coincides with his (Degen's) for *Glaucoma colpidium*. Nassonow, too, has decided that during systole in certain ciliates (*Epistylis, Campanella*), instead of the hitherto supposed complete disappearance of the vacuole, there remain behind several accessory vacuoles, which form, by their congress, the beginning of a new diastole. This is a different view from that which he takes of the behaviour in *Paramecium*. Nevertheless, they seem to behave alike.

The permanence of structure of the contractile vacuolar mechanism in *Paramecium* being admitted, it is not surprising that a pore first observed by O. Schmidt (1853) constituting the exit to the outside should exhibit evidence of equal permanence. Observation of the living animal shows this to be the case. The pore appears at proper focus as a bright granule surrounded with a darker ring, but it is scarcely distinguishable at first from any small granule. Zenker (1866) saw the same in *Bursaria*, Schewiakoff in *Frontonia* (through Calkins, 1901) and Wrzesniowsky (1869) in *Paramecium Bütschlii*. It can be determined by its position in regard to the disappearing vacuole at the end of systole when seen more or less *en face*, the last trace of the vacuole always lying beneath the pore; and by a radiate structure connected with the canaliculi, which can be seen until the close of the first period of diastole (contractile vacuole). The persistence of the radiate structure is itself strong evidence of the continuity of structure of the vacuolar apparatus. It arises from the fact that the proximal ends of the canaliculi dip downwards[2] into the cortex to make juncture with the contractile vacuole within the hemisphere lying against the cortex. Khainsky's figures support this description, as *e.g.* his Fig. 42. I cannot agree with this

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[1] Pütter's diagram (Fig. 3) is also susceptible of an interpretation different from that which he gives, and which Nassonow accepts.

[2] Not as Carter (1886) and Maupas (1883) thought, viz. in a position in the body more internal than the contractile vacuole.

author, however, that there is a depression ("invaginatio pulsatoria") of the surface above the contractile vacuole, such appearance being artificial, coming to view only in fixed material. In a strict profile view the pore appears *in vivo* as a clear line leading from the outer face of the contractile vacuole to the surface, but this is very difficult to see.

During the earlier period of diastole of the contractile vacuole that hemi-sphere of the same which lies in contact with the cortex maintains a conical form, the apex of the cone at the pore. This shape is taken under the urge of dis-tention caused by the flowing in of fluid from the canaliculi and is a sudden result of this distention. Following this is a longer second period of diastole during which the contractile vacuole becomes spherical, and the radiating structure disappears; that is, the radiant structure due to the proximal ends of the canaliculi. This struc-ture does not reach quite to the pore. Whether the pore is an opening or merely a place which tears open at each systole is hard to say. The latter is Khainsky's view "unwiderleglich festgestellt."

Spirogyra. Until recently it had been supposed that contractile vacuoles occur among plants only in zooids of various sorts, and in the border-line organisms. It is, however, now known that they occur in great numbers in the gametes of *Spirogyra* (Lloyd, 1924, 1925) during conjugation, during which period they constitute the mechanism by which the condensation of the gametes down to the volume of the definitive zygote is achieved. That this is no mean achievement is seen when it is known that the volume of the zygote amounts to *ca.* 20 to 40 per cent. of the total original volume of the gametes. The numbers and size of the contractile vacuoles are such as to render them objects of extraordinary interest, their chief disadvan-tage as biological material being the uncertainty of the supply.

The contractile vacuoles first appear in the gametes at about the time of fusion, that is probably just before, rather than just after, the act of fusion is accomplished, this being conditioned by the breaking down of the septum in the conjugation tube. Those first formed are very small and inconspicuous and may easily be overlooked, but when one is aware of the position in which they are to be sought they may then be found long before a measurable degree of condensation has taken place. This is, namely, in the cytoplasm and in the immediate vicinity of the conjugation tube. From this region as a centre, vacuolar activity spreads slowly toward the more distant regions of both gametes. The rate at which this activity is propagated through-out the gametes is indicated by a recent series of observations made on *S. porticalis*. The first contractile vacuole observed was in the cytoplasm of the conjugation tube near the septum in the female at 8 p.m. During the following hour a large number of similar vacuoles, not larger than 5 microns in diameter on bursting, were formed in this region, many of them in contact with the edge of the chloroplast, a fold of which jutted slightly into the tube. The first vacuole to form on the opposite side of the gamete from the tube was seen at 9.20, a distance of 30 microns away. The first one to form quite at the end farthest away, at a distance of 70 microns, was seen at 10 o'clock. In this case therefore it took about two hours for the whole cytoplasm to become involved. The septum broke down, effecting communication

between the gametangia, at about 9.50, at the time approximately when the entire gametal cytoplasm had become actively vacuolated.

After this time, the vacuoles increase both in size and in numbers. In size they frequently surpass the half diameter of the cell, and their number, during the whole period of conjugation, must be well above 100, probably being about 200 to 300. The looseness of the data is undeniable, but any reasonable error may be introduced without making the whole behaviour much less remarkable.

The locus of the contractile vacuoles appears at first undeterminate, but as one studies the matter, it becomes apparent that the majority arise along the anfractuous edge of, and in immediate contact with, the chloroplast, and there seems little doubt that this is a place where the formation of vacuoles proceeds with greatest ease. At the same time, however, similar vacuoles are formed in the cytoplasm removed from the chloroplast, so that the mechanism involved is usually but not necessarily associated with the chloroplast. It being considered by many that a "pulsating" vacuole is one which repeats itself (we have seen that it is only in such case that this adjective has any meaning), the question is often raised (*e.g.* by Pascher, 1927) if the contractile vacuoles in *Spirogyra* occur repeatedly in the same position. I am now able to answer this in the positive, for, by close observation, I have become convinced that vacuoles may be repeatedly formed in the same position exactly, and, what is more, burst repeatedly in the same point on the surface. But I do not venture to assert more than this, and what may be inferred from the facts is not certain.

Pascher (1927) has thrown doubt on the correctness of my conclusion because based on the alleged frequent pathological condition of "Deckglassmaterial." The criticism seems captious, but in order to meet it I have studied conjugating material with the utmost care, sparing it all unnecessary disturbance (just as did Czurda) and following the behaviour with an 8 mm. Watson apochromatic, *without cover glass*.

Several cases were studied from start to finish, not by myself alone, but by my associate, Miss J. Spier. In spite of the obvious difficulties I succeeded in making four series of photomicrographs of conjugating gametes, *without any cover glass*, the filaments lying undisturbed in their original position, the mat having been cut out with a sharp pair of scissors and mounted in the ditch water in which it grew. This was done immediately on securing the material from a nearby ditch which furnishes material in the spring of each year. The photographs (Figs. 6, 7) were made with a 8 mm. Watson apochromatic and Zeiss × 7 ocular, with a heat filter.

To some extent the contractile vacuoles in *Spirogyra* may be described as "roving." This becomes clear if the vacuoles in the region of the conjugation tube are watched during the period just following the breaking down of the septum, and before a relatively constant difference of pressure between the gametes is established, though it must not be forgotten that reversals of pressure and therefore of flow can take place throughout the period of conjugation. Just after the septum has disappeared, however, one may observe vacuoles from the male slipping through the port into the female, and back again, and those originally in the female behaving

similarly. The movement is of course purely passive, and in this respect the vacuoles are like those in *Amoeba*.
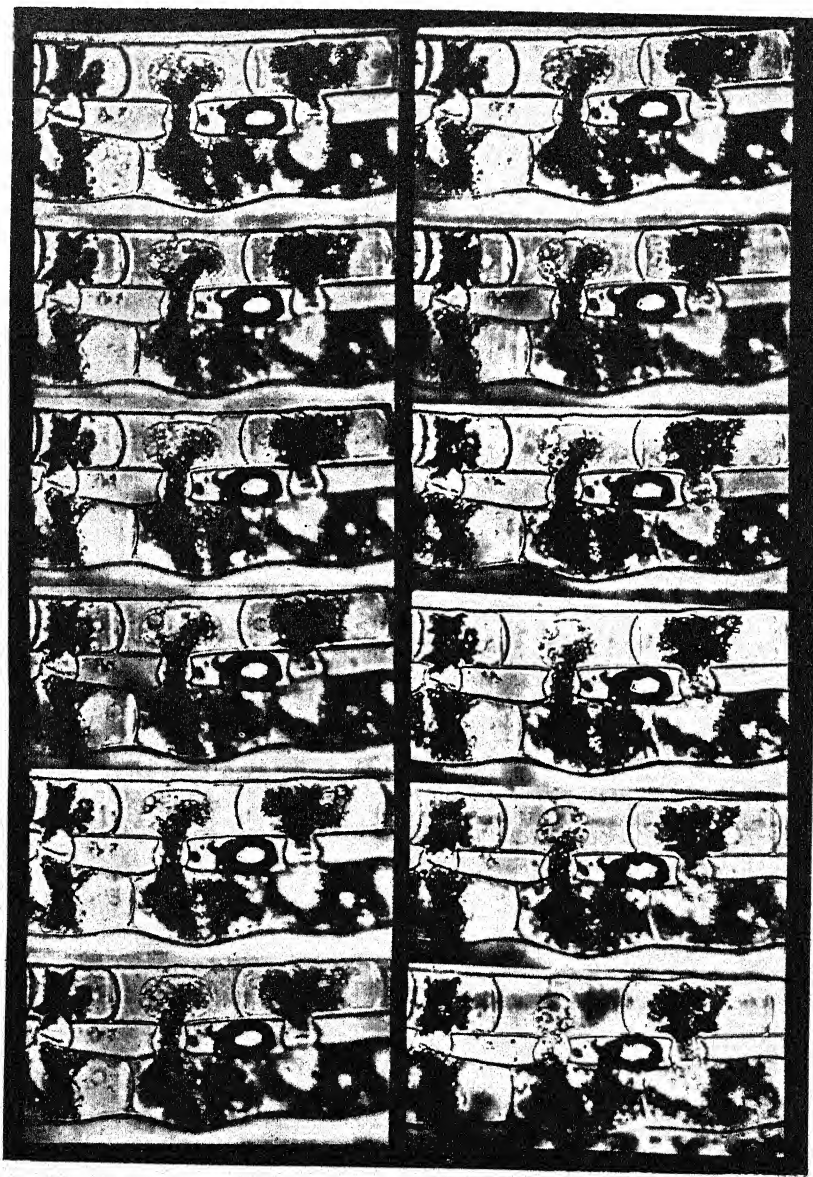

Fig. 6. Twelve photomicrographs in series taken during conjugation, *Spirogyra porticalis*, without cover glass.

Though the precise condition for the origin of contractile vacuoles is in doubt, there is none that the conditions under which they take up water are osmotic. I have

shown (1928) that the concentration of solution of the contents of the vacuoles is higher than that of the central vacuole, which during maturation is materially reduced, as Klebs long ago discovered. The change from higher to lower con-
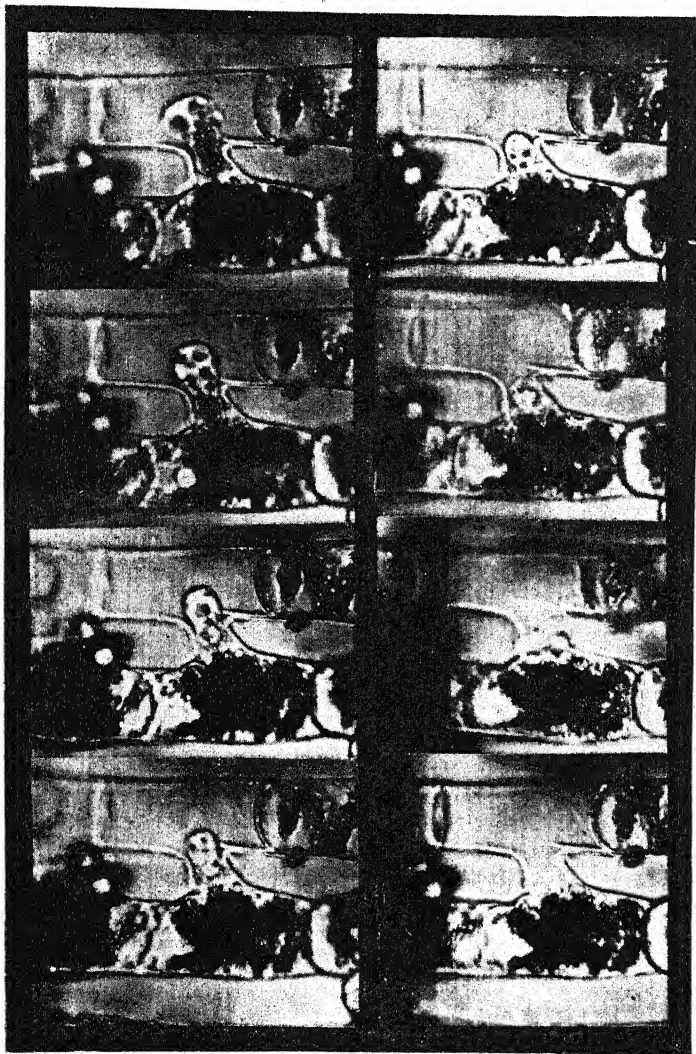

Fig. 7. Eight photomicrographs in series taken during late conjugation, *Spirogyra porticalis*, without cover glass.

centration of solution is progressive, and comes to fall in the male gamete to something below 0·1 N, while that of the contractile vacuoles is rather above 0·2 N. The inhibition of their activity follows the use of plasmolytes of higher concentration than this, and, in effectiveness, is proportional to the concentration used. We can

therefore take the position of Degen (1905) that the "contractile vacuole is originally a purely osmotic system."

Degen's hypothesis that with diastole the membrane becomes permeable is open to question. It appears to me that the very fact he cites in support of his view can be taken to mean the exact opposite, namely, the formation of secondary vacuoles, which ensues before systole. Khainsky has criticised Degen in a somewhat similar sense.

Stempell (1914) found a relation between frequency of pulsation and the concentration of the medium, animals in media of higher concentration showing a lower frequency of pulsation.

The peculiar locus of the contractile vacuoles in *Spirogyra* makes possible a further analysis of the condition under which they break outwardly. The peculiarity referred to is the fact that, in *Spirogyra*, the contractile vacuole occurs within an extremely thin layer of cytoplasm, probably, in many instances, not thicker than 2 microns. In consequence the contractile vacuole is literally a bubble of cytoplasm lying appressed against the cell wall, and jutting out into the central vacuole, having the shape of a soap bubble lying on a rigid surface. The greater extent of surface lies in contact with the fluids of the central vacuole. That the contractile vacuoles burst outwardly and not inwardly can hardly be due to any other condition than that of surface tension, which I have argued is lower at the cytoplasm/central vacuolar fluid than at the cytoplasm/cell wall fluid interface. The mutual bursting of two contingent vacuoles, which indeed occurs, is understandable on the same grounds. We may extend this explanation to other forms for it would seem that the conditions stated can hardly be otherwise. Bütschli indeed (1887–9, p. 1432) appreciated the possible rôle of surface tension, but he attributed a higher surface tension to the small vacuolar drop in comparison to that of the outer surface of the organism against the medium.

A recent observation made by myself on *S. porticalis* seems to throw some, if uncertain, light on the behaviour of the substance which acts, as described by Lloyd and Scarth (1926), as if it were clothed with lecithin (or some substance which forms myelin bodies). The behaviour was so clear and unequivocal that I do not hesitate to describe it, though the interpretation is another thing.

It is of course well known that during conjugation many gametes enter, for one reason or another, into a pathological condition; I mean by this, any condition which is aberrant or abnormal in any way. Of a great variety of behaviour in this regard the following is a case in point.

Four pairs of gametes were found conjugating, but somewhat abnormally, as the cytoplasmic utricles of the males were not contracting synchronously with the chloroplast, in fact they were lagging behind. As I watched them, in two of the males the endoplasm left the ectoplasm and continued to condense by means of contractile vacuoles, the ectoplasm apparently being at a standstill. A most curious fact now emerged, namely, that the endoplasm slid over and withdrew from some of the contractile vacuoles before they burst, and these were accordingly left as contractile vacuoles of the ectoplasm alone. The ectoplasmic membrane and the attached

vacuoles had the same optical appearance, apparently structureless, double-contoured and homogeneous. The contractile vacuoles slowly enlarged and burst, and it was observed by me that additional new ones were formed and burst, in consequence of which (in part) the *ectoplasmic utricle also condensed* but at a much slower rate than the endoplasm. The sap of the central vacuole (enclosed by the endoplasmic membrane) contained many granules in lively Brownian movement; that of the space between the endo- and ectoplasm was almost free of granules. These observations lead to the inference that the contractile vacuoles are of ectoplasmic origin.

A second observation was the following. As the endoplasmic membrane decreased in volume (contracting toward the port of the conjugation tube), the external free surface became active. This activity was first observed as a lot of minute eruptions—cytoplasmic processes—which were put forth and withdrawn with considerable speed. Soon the ends of the slender processes broke up and a lot of rounded vacuoles, each containing *ca.* 2 to 4 granules which vibrated freely within, hitting constantly on the wall and causing the membrane to bulge. But the membrane itself was highly mobile, constantly changing its shape. In the course of a short time the vacuoles might run together to form a single vacuole, which then would show remarkably vigorous movement; or the loosened portion might again become attached to the original membrane and disappear, to reappear later. So far as I could observe there was no growth in size of the vacuoles, their osmotic pressure appearing therefore to be the same as that of the surrounding fluid (within the ectoplasm)[1].

The bearing of these observations is too far reaching to be hastily indicated. One inclines to say that the active cytoplasm has widely different properties from those of the membranes, and are such as characterise lecithin in water, elsewhere referred to by Lloyd and Scarth.

V. THE FUNCTION OF THE CONTRACTILE VACUOLE.

It is probably a fair assumption that the contractile vacuole has the same function in all the protozoans and similar forms in which it occurs. This at all events seems to be implied by the workers in the field. It is this assumption that would allow us to carry over to other forms the experimental results obtained by working on the largest and most favourable objects for study, such as *Spirostomum*, *Paramecium*, etc.

The assumption that this " organelle " has only one function is perhaps questionable, and it is possibly this which has led us more or less astray in looking for an answer to the question of what the function is.

Historical presentations of the various views which have been held are sufficiently numerous and available so that we need not treat this matter at great length. The most recent such review is by Weatherby (1927). One should consult the general works cited on page 329.

That the contractile vacuole is a spermatic gland was the first attempt at an

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[1] There was certainly no evidence of parasitism.

explanation by Ehrenberg in 1838, and is sufficiently beside the mark as to go into the joke column and thus lighten what might otherwise be a heavy performance.

I have already adverted to the ideas of Müller, and of Claparède and Lachmann, that the contractile vacuole and canals in *Paramecium* are a circulatory system, an idea connected with their conviction that there is no opening to the exterior. Lieberkühn (1856) is always placed with the above authors as agreeing with them, but I think not wholly fairly, since he could not agree that there was a movement of fluid back from the contractile vacuole into the canals. He says: "I have never yet found in any Infusorium special canals in which the fluid is seen to flow back into the body during the systole, and which would give the means of a perfect circulation." He says this under the caption "The Vascular System" it is true, but a careful reading of his paper shows that he was bowing to a general belief rather than accepting it. Also he opposed Oskar Schmidt in his belief that there was an external opening. Had he sided with him in this, the rest would have been easy, and he would have verged away from the conclusions in the Genevese memoire. It is worthy of remark further that Müller, Lieberkühn, Claparède and Lachmann, and Spallanzani had sound observation to their credit, namely, that the enlargement of the canals precedes the evacuation of the contractile vacuole. Since Spallanzani (1777) was the first to record this observation, his words are worth quoting. "A toutes les trois ou quatre secondes, les deux petits globes centraux se gonflent comme des utricules et deviennent plus gros du triple ou du quadruple, et l'on aperçoit le même changement dans les rayons des étoiles, avec cette différence, que lorsque les petits globes s'enflent, les rayons désenflent[1]." Spallanzani thought the apparatus of respiratory nature, this arising out of his idea of a tidal movement of fluid between central, contractile vesicle and the canals. The two conceptions above mentioned are not mutually exclusive, but there is at present no evidence one way or another, if the view which Lloyd and Beattie advocate is correct, and which in part substantiates Müller, Claparède and Lachmann, and Spallanzani, and agrees with the description of Stein.

Siebold and Stannius (1854, 1858) support these authors in saying that the vacuoles constitute a circulatory system, "the first attempt at a circulation of nutritive fluids," but their statements are too summary and loose to permit the extraction of a definite description of behaviour. Samuelson (1857) was heart and soul with Claparède and Lachmann, but I do not find that he afforded any evidence *pro* or *con* except the tacit admission that there is no external opening.

As long as this position was maintained the above views reigned, viz. either that the "organelle" in question is circulatory or respiratory or both, but when it was admitted that there is an escape of fluid to the outside of the body, the various people who thought about the matter became excretionists in one form or another. Either the fluid expelled contained urea or something of the kind, or was merely water, with chance or unavoidable mixtures (Bütschli), but expelled merely to keep

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[1] Spallanzani, Lazare, *Opuscoli di fisica animale e vegetabile*, Modena, 1777 or 1776. Not having seen the original paper, I am relying on the French translation, cited and quoted by Dujardin in his *Histoire nat. des infusoires*.

the water content of the animal at approximately a constant. The first serious protagonist for the former of these was Griffiths (1888–9, p. 131) who believed that he was able to detect uric acid in the vacuoles by means of the murexide test, and adopted the view that the vacuole is a kidney. Howland (1924) was, however, unable to verify his results with the same method, but on using the Benedict blood-filtrate test she believed that she was able to prove that uric acid is eliminated in some way. As the test, however, was applied on masses of organisms, she could not press the matter further. To do this Weatherby (1927) first tested cultures of previously washed *Paramecium* for ammonia (by Nesslerisation) and found that in cultures thirty-six or more hours old, ammonia was always present, from which he concluded that either ammonia was excreted as such or occurred as the hydrolysis product of some other excrete. He then pressed the matter further by injecting Nessler's reagent by means of Taylor's micro-pipette, succeeding in injecting the reagent twelve times into contractile vacuoles. Three times the reagent was injected into the cytoplasm surrounding the vacuoles. In no case, however, did Weatherby succeed in getting the positive reaction. By adding urease to the culture fluid he obtained positive evidence of the occurrence of urea, in which form he argues nitrogen must be, in part at least, eliminated by the animal. He then applied the xanthydrol precipitation test for urea by injecting[1] the reagent into the contractile vacuoles as before. No trace of the characteristic needle-shaped crystals of di-xanthyl urea was found in any case, from which it is argued that the concentration of urea, if present in the contractile vacuole at all, is too low to be detected. By a calculation based upon Maupas' figures (that the quantity of water evacuated by the contractile vacuole in 46 minutes, at 27 degrees, is equal to the total volume of the organism), Weatherby found that the quantity of urea which should occur in the fluid of the vacuoles should be about one part in 2000–3000. Inasmuch as the reagent is sensitive to one part in 12,000, it was concluded that the vacuoles play no part in the excretion of urea. This author then favours the theory that the function of the contractile vacuoles is to regulate the hydrostatic pressure within the cell.

This is the theory which was first advanced by Hartog (1888–9) at the meeting of the British Association for the Advancement of Science. His argument ran as follows:

Contractile vacuoles are present in naked plant zoospores, *Protozoa* etc. except in those which are parasitic or marine. The failure of the contractile vacuoles to act, as occurs under untoward conditions, results in dropsical animals. When plant protoplasts are extruded into water the protoplasm becomes vacuolated, and the vacuoles burst, which does not occur if a sufficient quantity of sugar, saltpetre, glycerine or other innocuous substance is added in suitable concentration. This occurs because protoplasm contains in its interstices substances of high osmotic value, so that on exposure to water unprotected by a membrane to support its surface, it of necessity acts thus. Naked protoplasts must then have a mechanism

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[1] It is interesting to note that Ogden Rood read a paper before the Berzelian Society of Yale University in 1852, in which he suggested the possibility of injecting reagents into the body of *Paramecium* (Rood, 1853).

to rid themselves of excess water, and this is the contractile vacuole. Hartog then makes the suggestion, which I myself made (Lloyd, 1926) in ignorance at the time of Hartog's contribution, that many secretory cells in the more differentiated forms may function by means of these "organelles."

Those who have supported Hartog's opinion, and have advanced experimental evidence therefor, are Degen (1905), Burian (1910), Zülzer (1910) and Stempell (1914)[1]. There is at the present time a rather general acceptance of the contractile vacuole as a hydrostatic organ, as indicated by the repeated statements and implications in Calkins' treatment of the Protozoa (1909, 1926).

It is generally supposed that the absence of contractile vacuoles from marine and parasitic forms is in harmony with this view. But the phagocytes of the peritoneal exudate of *Salamandra* form contractile vacuoles (Klemensiewicz, 1903, p. 63) under conditions which are not normal, but do not involve a lowering of the concentration of the medium. Klemensiewicz regards them as normal responses to changed biological conditions. Margaret Zülzer (1910), having noted the absence of contractile vacuoles from marine protozoans, found that there occurred a complete abatement of the contractile vacuole in *Amoeba verrucosa* which she accustomed to sea water by slow evaporation during several weeks, to return again on the reverse change of salinity.

Miss Zülzer refers to Yasuda's (1900–1) work, but I cannot find that he had anything to say about the behaviour of the contractile vacuoles. He says that the "vacuoles" become larger and more numerous under increasing concentrations of the medium, but it is evident from his figures that he is referring to food vacuoles. His figures of *Paramecium* show no differences in the contractile vacuoles, but do show differences in the food vacuoles. The form of the argument is clear, but it happens that the fact seems to be that some marine forms do have such vacuoles, and it would seem that further investigation of these organisms would throw more light on the problem.

In *Spirogyra*, as I have shown, the function of the contractile vacuoles is unequivocally to rid the gametes of water (held in the central vacuoles), leading to their condensation to the volume of the definitive zygote. The process is assisted by the circumstance that the osmotic pressure of the sap of the central vacuoles of the gametes becomes gradually reduced during the later phase of the maturation period, though this of itself is not sufficient to permit reduction of volume appreciably, and certainly not enough for the free play of surface tension at the surface of the gametes, especially the male, sufficient to ensure complete, or even measurable, condensation, as Czurda (1925) has suggested. It is the reduction of volume through the activity of the contractile vacuoles which effectually permits this.

One is tempted, therefore, to compare the whole lot, assimilating them on a common ground by saying that the whole function of contractile vacuoles is to get

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[1] An example of this behaviour may be found in the method of secreting caoutchouc in *Parthenium argentatum*. This substance originates as fine suspensoids (3 μ or less in diameter) in the cytoplasm of the parenchyma, but is thrown out into the sap, where it accumulates until it forms a dense, latex-like emulsion. The suspensoids must be ejected through the surface of the endoplasm into the central vacuole. I have referred elsewhere to Gurwitsch's and to Ludford's interesting studies.

rid of water in those forms which have not the support of a membrane of sufficient rigidity to resist the osmotic pressure of the protoplast. This might, however, be going too far, especially as we believe to have shown that there is after all, in *Paramecium*, something like a circulation (as some of the earlier authors thought of it) or better, in addition to a discharged volume of fluid, there is a tidal flow of fluid from the contractile vacuoles into the canals and back again (Lloyd and Beattie). It may be rather more wholesome to realise that we probably do not know enough about the objective facts yet than to speculate on their meaning as we now suppose them to be, except, perhaps, in so far as we seem to have some positive experimental knowledge as to what the organelle in question does not do (Weatherby).

It has not yet been determined whether Schewiakoff (through Calkins, 1926, p. 169) was right in thinking that the products of dissolution of the crystalline calcium phosphate seen in *Paramecium* are carried off by the contractile vacuoles.

Efforts have been made from the biophysical point of view to procure models.

Quincke's experiment on drops of air and oil in the presence of added reagents which cause periodic changes in the surface tension of the interfaces leading to pulsatory changes in volume, while of importance from the point of view of periodicity, do not seem to bear upon the behaviour of contractile vacuoles, except as they indicate that the conditions which surround such vacuoles may affect their volume within narrow limits. He entertained a rather too obvious inference from his experiments that contractile and other vacuoles were clothed by an oil layer. We have seen that Nassonow believes that there is a layer clothing the vacuolar apparatus of *Paramecium* which yield to osmication, becoming blackened. But whether this lipoid material plays a part in procuring contraction is a question which cannot be answered.

Rhumbler (1898) made models with drops of (a) glycerine and castor oil in 70 per cent., (b) colophonium dissolved in oil of turpentine in 70 per cent., and (c) chloroform in water. From such systems droplets are thrown off into the surrounding medium. The bursting of the "excreted" droplets which appear depends upon the lowering of their specific surface tension. Rhumbler regards the character of the membrane (*Amoeba*) which constitutes the vacuolar wall as determined by the nature of the watery fluid which contains solutes taken up from the protoplasm, and that it is necessarily different from the extoplasmic medium interface.

Stempell (1914) constructed an ingenious, if somewhat awe-inspiring complicated piece of apparatus, a description of which must be omitted, which acted as he conceived the vacuolar apparatus in *Paramecium* to do, that is to say, a periodic emptying of a "vacuole" took place accompanied by the emptying of the "canals." Fundamentally the apparatus was an osmotic system, its activity depending on the difference of concentration of the fluids of the surrounding medium, of the "protoplasmic body" and of the "vacuole." The actual periodicity depended on the action of valves. If, as may be objected, this apparatus does not provide an exact analogy to the apparatus of *Paramecium* in view of Stempell's mistaken (in this paper so regarded) view of the way in which it acts, a little more ingenuity

might result in modifications of Stempell's model which would perfect the analogy. The point is that the apparatus did what Stempell meant it to do.

Note while in press. During the past two weeks the writer has been engaged at the Biological Station, St Andrews, N.B., in examining marine protozoa, and has found a number of species in which the contractile vacuole occurs, and its periodicity has been determined. In a few species it has been difficult to see, if present. The facts seem not to harmonise with the generally accepted statements of the texts. The details of the study will be published elsewhere.

St Andrews, N.B.
12 *Aug.*, 1928.

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